UNDERSTANDING THE INNATE IMMUNE RESPONSE IN VIRAL HEPATITIS

INTERFERONS AND NK CELLS

Rik Anton de Groen

The studies described in this thesis were performed at the Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands.

ISBN/EAN: 978-94-6233-750-3
Cover: Houston, Sam. *Innate*. 2014. Oil on canvas.
Printed by: Gildeprint

Understanding the Innate Immune Response in Viral Hepatitis

Interferons and NK Cells

Inzicht in het Aangeboren Immuunrespons in Virale Hepatitis

Interferonen en NK Cellen

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

dinsdag 10 oktober 2017 om 13.30 uur

Rik Anton de Groen
geboren te Rochester, Minnesota
de Verenigde Staten van Amerika

Erasmus University Rotterdam

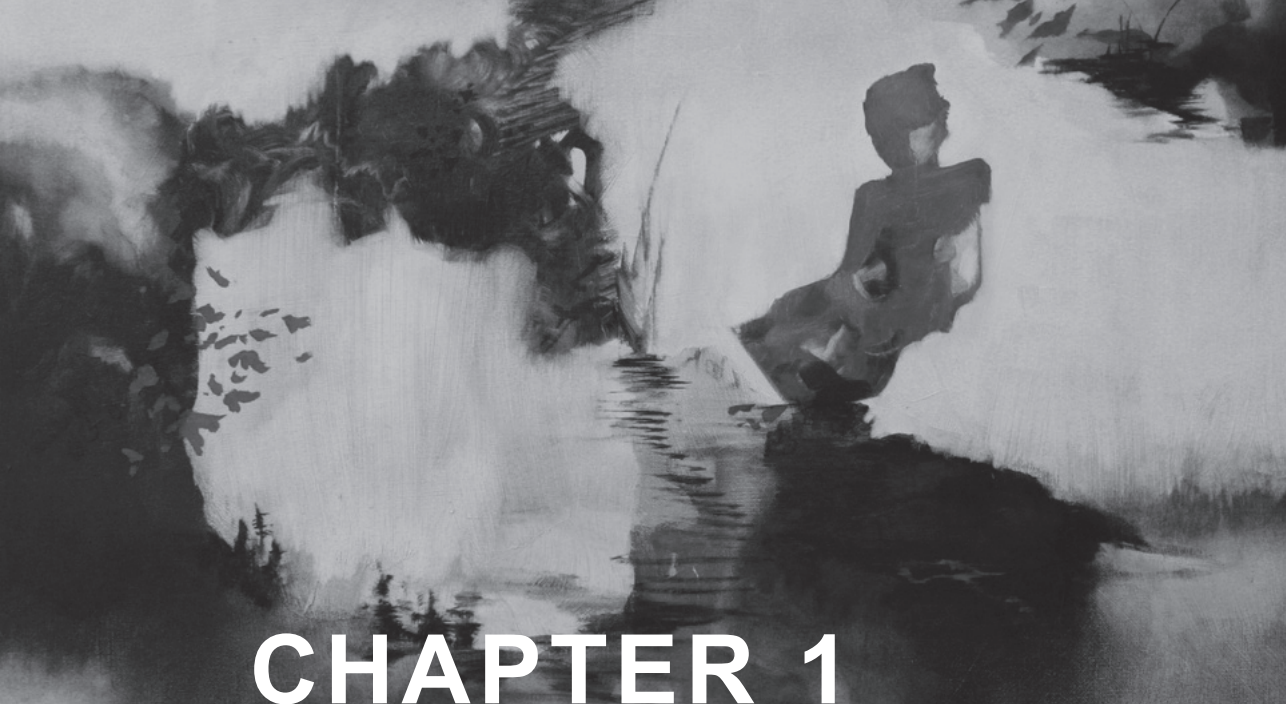
Promotiecommissie:

Promotor: Prof.dr. H.J. Metselaar

Overige leden: Prof.dr. C.C. Baan
Prof dr. C.A.B. Boucher
Dr. J.E. Arends

Copromotor: Dr. A. Boonstra

TABLE OF CONTENTS



CHAPTER 1

INTRODUCTION AND OUTLINE OF THE THESIS

VIRAL HEPATITIS INFECTION

Viral hepatitis, a group of diseases caused by infection of preferentially hepatotropic viruses, is the leading cause of liver inflammation worldwide. There are an estimated 400 million people affected by viral hepatitis infection, with 6–10 million new cases of infections reported annually.[1] Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the two most prominent forms of viral hepatitis, with 240 million and 130–150 million individuals chronically infected respectively.[2,3] Acute HBV infection is spontaneously resolved in over 90 % of infected adults, but if not resolved results in a chronic persistent infection, particularly in neonates that contract the infection through vertical transmission.[4] This is in stark contrast to acute HCV infection, where only 15–25 % of adults infected spontaneously clear the virus without treatment and the majority progress to chronicity.[5,6]

Prolonged infection with HBV and HCV results in a state of chronic liver inflammation, and consequently a significantly increased risk of developing liver cirrhosis and/or hepatocellular carcinoma. Every year, an estimated 686,000 and 700,000 people die due to complications related to HBV and HCV infection respectively.[7] Despite significant advances in prevention and treatment, including a prophylactic HBV vaccine and highly effective direct-acting antivirals (DAAs) against both HBV and HCV, these two diseases still pose a massive disease burden globally, with HCV-related end-stage liver disease still being one of the leading causes of liver transplantation in the United States.[8]

Hepatitis B Virus

HBV is a member of the Hepadnaviridae family of viruses, with a partially double-stranded DNA genome held in a relaxed circular formation (RC-DNA) by overlapping 5' ends of the two DNA strands. The genome is approximately 3200 base pairs in length and encodes a total of 7 proteins; the pre-core protein (hepatitis B envelope antigen, HBeAg), core protein (HBcAg), 3 surface proteins (HBsAg), X protein (HBxAg), and a DNA polymerase. Collectively, these proteins comprise the 42 nm viral particle that consists of a protein nucleocapsid core, composed of HBcAg, which surrounds the viral genome and polymerase, as well as a lipid envelope embedded with surface proteins (HBsAg). HBxAg, a non-structural protein, is required for the efficient transcription of covalently closed circular DNA (cccDNA), a plasmid-like episome that functions as a template for viral RNAs, and consequently new virions. The non-particulate HBeAg, generally located between the nucleocapsid core and lipoprotein envelope, is secreted and accumulates in the serum as a soluble antigen. Replication of HBV can be divided into 3 mains phases; (I) the release of the RC-DNA-containing nucleocapsid into the cytoplasm and transport of RC-DNA to the nucleus for repair and formation of cccDNA, (II) transcription of cccDNA to form viral RNAs, (III) and encapsidation of

these RNAs, together with the DNA polymerase, for reverse transcription of new RC-DNA and consequently formation of infectious virions.[9,10]

The 8 major genotypes of HBV, denominated A-H, show different geographical distribution, with A/D being the predominant genotypes found in Europe and B/C in Asia. Contrary to HCV infection, chronic HBV infections are characterized by disease episodes with differentiating serum levels of HBV DNA, alanine transferase (ALT), a marker of liver damage, and HBV HBeAg. Using these parameters, different clinical phases have been discerned to describe the dynamics of the natural history of chronic HBV infection, and determine the indication for antiviral treatment on the basis of rate of viral replication and ALT elevation. Chronic HBV patients have been categorized into 4 clinical phases: the HBeAg-positive immune tolerant (IT) and immune active (IA) phases, as well as the HBeAg-negative inactive carrier (IC) and hepatitis (ENEG) phases (Figure 1). Currently, the European Association for the Study of Liver Disease (EASL) recommends 2 mains strategies for the treatment of chronic HBV infection; treatment for a finite period with PEGylated interferon-α (IFN-α) or a nucleot(s)ide analogue (NA), such as entecavir and tenofovir, or long-term treatment with NA(s). The main advantage of IFN-α treatment is the potential for long-term systemic control of HBV infection off therapy with an opportunity to obtain a sustained virological response (SVR), while NAs have been shown to be highly potent inhibitors of HBV replication, have a high barrier to resistance, and induce few off-target effects but demonstrate low levels of SVR off treatment.[11]

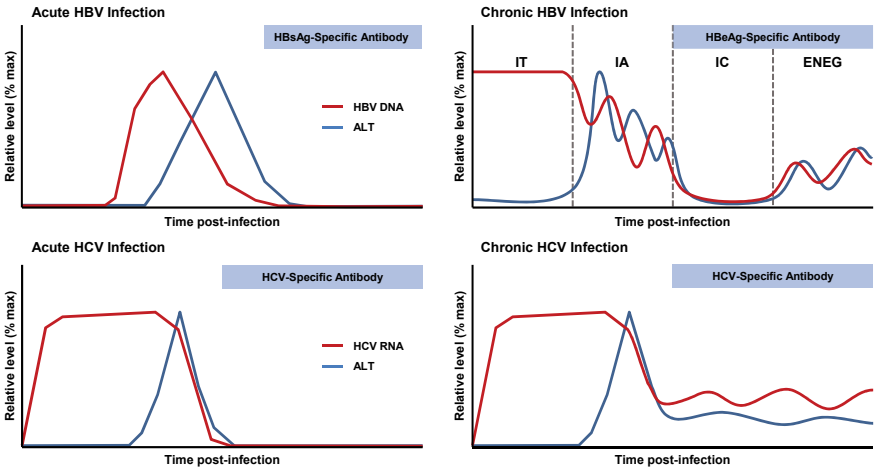


Figure 1. Natural course of disease for acute and chronic viral hepatitis infections.

Schematic presentation of viral load (HBV DNA and HCV RNA) as well as alanine transferase (ALT) levels, a marker of liver damage, in the serum of acute or chronically infected HBV and HCV patients. Typical time courses for acute viral hepatitis infections range from weeks to months until resolution, while progression of chronic hepatitis infection occurs over significantly longer periods (years). HBV clinical phases: immune tolerant (IT), immune active (IA), inactive carrier (IC), and HBeAg-negative hepatitis (ENEG).

Hepatitis C Virus

HCV is a member of the Flavivirus family of viruses, with a small positive-stranded RNA genome. The viral particle is approximately 50–55 nm in size and consists of an icosahedral protein core encased by a lipoprotein envelope. The viral proteins comprising these structures, and other non-particulate proteins, are encoded by the 9600 base pair genome and can be classified into 3 major groups; core protein, envelope glycoproteins (E1 and E2), and non-structural proteins (NS2, NS3, NS4A/B, NS5A/B, and p7). The structural proteins E1 and E2 are found as dimers anchored to and radiating from the viral envelope, while HCV core proteins compose the nucleopcasid surrounding the viral genome. HCV replication begins after the binding and internalization of the virus by a hepatocyte via endocytosis. HCV RNA is released into the cytoplasm, and the viral RNA is translated into one large polyprotein at the endoplasmic reticulum. The NS3 protein, together with NS2 or NS4A, form the NS2–3 cysteine protease and NS3 serine protease/NS4A cofactor complexes that are responsible for the cleavage of functional proteins from the HCV polyprotein. Once the functional proteins have been cleaved, RNA replication can be facilitated by the RNA-dependent polymerase NS5B and the viral components are assembled into an infectious HCV virion that is later released from the hepatocyte. The NS5A protein has no enzymatic activity, but is essential for proper viral RNA replication and modulates cell-signaling pathways. Although the exact roles of membrane proteins NS4B and p7 remain more ambiguous, they have also been demonstrated to be important in viral replication and formation of infectious virions respectively.[12,13]

HCV has 6 major genotypes (designated 1–6) and more than 50 different subgenotypes. The absence of an HCV proofreading exonuclease and subsequently high nucleotide error rate during RNA replication likely explains the large genotypic variation. Recent advances in DAAs have completely shifted the landscape of chronic HCV therapy from 24–48 week PEGylated IFN-α/ribavirin centered treatment strategies (54–56% SVR in patients)[14,15] to shorter 8–12 week, more tolerable, and highly efficacious DAA regimens (94–99% SVR in treatment naïve and experienced patients).[16–18] DAAs, such as sofosbuvir, daclatasvir, and ledipasvir, directly inhibit different HCV functional proteins (e.g. sofosbuvir NS5B and daclatasvir/lepidasvir NS5A) with limited off-target effects and have therefore become the recommended treatment strategy by the EASL.[19]

INNATE IMMUNE RESPONSE IN VIRAL HEPATITIS

Infection with a virus triggers a series of diverse immune responses in the host in attempt to interrupt and eradicate virus while minimizing damage to the host itself. In viral infections, such as HBV and HCV, the cumulative effectiveness of these host immune responses is the determining factor in successfully clearing

the virus or developing a chronic persistent infection. Traditionally the mammalian immune system and response has been divided into two major categories, the innate and adaptive immune systems. The innate immune response is evolutionarily conserved among multi-cellular organisms and represents the first line defense and immediate response against infections, while the adaptive immune system provides a more versatile means of specific of defense against pathogens and protects the host against subsequent reinfection with the same pathogen.

Cells of the innate immune system recognize common antigens called pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) such as toll-like receptors (TLRs).[20,21] The recognition of antigens initiates the innate immune response, leading to the upregulation of major histocompatibility complex (MHC) classes I and II as well as their respective co-stimulatory molecules, in addition to the secretion of inflammatory cytokines that more efficiently prime and subsequently initiate the adaptive response.[20,22] During HCV infection, cytosolic viral RNA is sensed by retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), as well as double-stranded RNA (dsRNA) by TLR3 in endosomes, leading to IFN-mediated responses and the eventual production of various types of IFNs that suppress HCV replication.[23,24] In contrast, HBV infection seems to lack an induction of IFN-responses and HBV is only limitedly recognized by the innate immune system.[25,26] These contrasting inductions of the innate immune response can partially be attributed to a difference in viral kinetics; HCV is characterized by a rapid increase in serum RNA levels immediately after infection, while increases in HBV DNA levels are delayed, with the exponential phase of HBV replication only occurring weeks after infection.

Interferons

As previously referenced, IFNs are one of the key components of the innate immune response to viral infections. IFNs, described to have anti-viral, anti-proliferative, and immunomodulatory effects, are divided into three majors classes; type I, II, and III IFNs, each with unique complimentary receptor complexes through which they signal. Type I IFNs, the most prominent and well-studied class, is comprised of 13 partially homogenous IFN-α subtypes, IFN-β, as well as the more poorly defined IFN-δ, IFN-ε, IFN-ζ, IFN-κ, IFN-τ, and IFN-ω.[27] IFN-α/β production has been described for a large range of cells in the body, but specific immune cells, such as plasmacytoid dendritic cells (pDCs), have been shown to be especially potent producers of type I IFNs.[28,29] Type II IFNs are composed of only one cytokine, IFN-γ, and signal through its own unique IFN-γ receptor, a tetramer of IFN-γR1 and IFN-γR2 complexes, expressed on a broad range of cell types.[30] IFN-γ is primarily produced by natural killer (NK) and T cells. Lastly, type III IFNs, comprised of IFN-λ1, IFN-λ2, IFN-λ3, and IFN-λ4, have received increased atten-

tion in recent years, especially after the discovery of polymorphisms within the IFN-λ gene locus that are associated with spontaneous as well as IFN-α/ribavirin therapy-induced clearance of HCV infection.[31–35] Emerging evidence points to pDCs and a specific subset of BDCA3-expressing myeloid DCs (mDCs) as the primary immune cells capable of producing type III IFNs, while hepatocytes have also been shown to express and produce IFN-λ messenger RNA (mRNA) and protein upon HCV infection.[36–38]

Type I and III Interferon Signaling

Unlike type II IFNs, types I and III IFNs are potent inducers of an antiviral responses in infected and still uninfected cells to block viral replication and spread of infection. The similar antiviral effects of type I and III IFNs can partially be explained by comparable Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling cascades triggered upon IFN-α and IFN-λ receptor engagement (Figure 2). For both types of IFNs, engagement of their respective receptors results in activation of receptor-associated tyrosine kinases JAK1

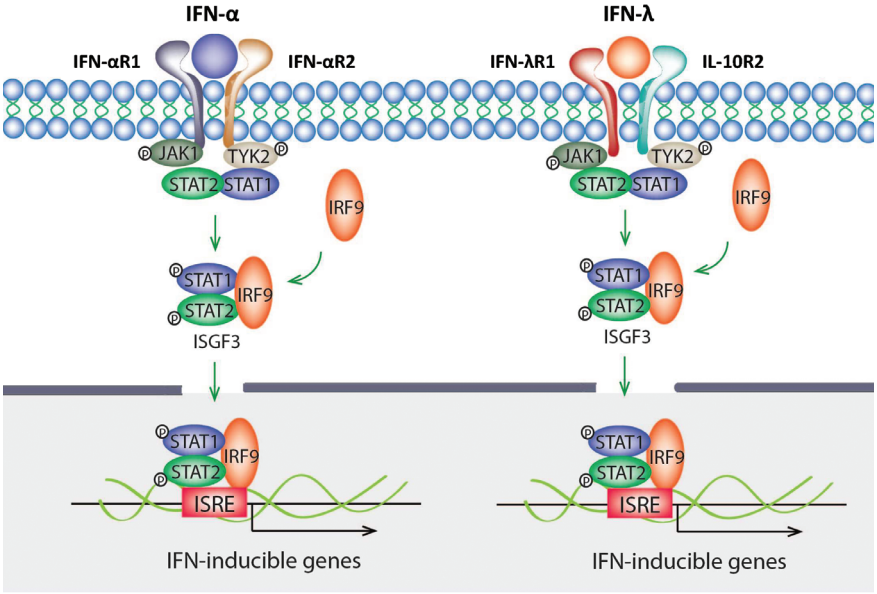


Figure 2. Type I and III IFNs are distinguished by their unique complimentary receptor complexes through which they signal.

IFN-α and other type 1 IFNs, engage through the IFN-α receptor complex, composed of IFN-αR1 and IFN-αR2, while IFN-λ signals through the IFN-λR1 and IL-10R2 complex. Despite triggering distinct receptor complexes, the downstream signaling of both IFN-α and IFN-λ is regulated through JAK/STAT signal transduction, ultimately resulting in the induction of IFN-stimulated response elements (ISRE) and initiation of ISG transcription.

and tyrosine kinase 2 (TYK2), leading to the recruitment of signaling molecules STAT1 and STAT2. This is followed by the phosphorylation of STAT1 and STAT2 and recruitment of IFN regulatory factor 9 (IRF9). Together with the dimerized STAT1 and STAT2, IRF9 forms IFN-stimulated gene factor 3 (ISGF3) complexes that bind promoter sequences in DNA ultimately resulting in the initiation of gene transcription.[39–41]

All members of the IFN-λ family use a specific transmembrane receptor heterodimer, the IFN-λR1 and IL-10R2 complex, which structurally differs from the IFN-α receptor complex, a heterodimer of the IFN-αR1 and IFN-αR2 subunits. In contrast to the ubiquitously expressed IFN-α receptor, the distribution of the IFN-λ receptor is more limited. Murine models have shown that epithelial cells express the receptor complex and respond to IFN-λ stimulation by eliciting an antiviral response.[42] Outside non-hematopoietic cells, specifically epithelial cells and hepatocytes, expression of the IFN-λR1 chain in humans has only been well described on pDCs, fibroblasts, and macrophages.[43–45] Many of the IFN-stimulated genes (ISGs) (e.g. myxovirus resistance 1 (MX1) and 2'-5'-oligoadenylate synthetase (OAS)) transcribed after engagement of type I and III IFN receptors are similar and function to induce an antiviral state within the cell. However, specific differences have been described for the signaling of IFN-α and IFN-λ. One example of this is the direct interaction between IFN-αR2 and IFN-induced protein ubiquitin specific peptidase 18 (Usp18) that inhibits further response to IFN-α, thereby creating a negative feedback loop.[46,47] This negative feedback loop is reflected in the shorter duration of immune response by IFN-α stimulation when compared to the more prolonged response observed by IFN-λ in epithelial cells and hepatocytes.[48–50]

To avoid detection by the innate immune system, both HBV and HCV have developed several strategies to interfere with the induction of IFNs.[51] The HBV polymerase is able to block TLR3 and RIG-I induction of IRF activation, inhibit stimulator of IFN genes (STING)-stimulated IRF3 activation, and inhibit IFN-α-induced nuclear translocation of STAT1.[52–54] In addition, HBxAg has been shown to downregulate the expression of mitochondrial antiviral signaling protein (MAVS), a downstream mediator of protein kinase R (PKR)-induced ISG expression.[55] In HCV infection, NS3-NS4A serine proteases cleave MAVs and Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF), a downstream mediator of the TLR3-induced signal transduction.[56,57]

Effects of Type I and III Interferons on Immune Cells

Type I IFNs have been shown to be strong modulators of multiple immune cell populations, including antigen presenting cells (APCs), such as DCs, monocytes, and macrophages (and their downstream promotion of T cell responses), as well as B cells and NK cells. However, due to the differences in IFN-α and IFN-λ

receptor prevalence and their respective signaling kinetics, the effects of IFN-λ on immune cells still remain controversial with limited evidence for many of these populations.

IFN-α has been previously demonstrated to have an activating effect on APCs, and enhances the expression of MHC and co-stimulatory molecules, such as CD80 and CD86, on the surface of DCs; this transiently increases the ability of DCs to stimulate T cells.[58–60] APCs are also potent producers of interleukin 12 (IL-12), a crucial factor for driving T helper 1 (Th1) responses during viral infections and an important factor in inducing IFN-γ production of T cells and NK cells. Although IFN-α has been shown to enhance bioactive IL-12 (IL-12p70) production in combination with TLR activation,[61] inhibitory effects have been described for the production of IL-12 subunit p40 (IL-12p40) by APCs.[62,63] This is in contrast to the effects of IFN-λ described on specific APCs. The stimulation of monocyte-derived macrophages with IFN-λ, in combination with activation via specific TLRs, resulted in an enhancement of macrophage IL-12p40 production whereas IFN-α inhibited TLR-induced IL-12p40 production.[43] IL-12p70 was, however, not produced by these stimulated macrophages without the addition of stimulatory signals, such as CD40 ligation or IFN-γ, required for the production of bioactive IL-12.[64–66] APCs that produce IFN-λ, such as pDCs, have also been demonstrated to be stimulated and modulated by type III IFNs in an autocrine loop.[37,45]

B cells play an important role in the resolution of viral infections through the production of neutralizing antibodies. IFN-α exposure of B cells strongly promotes humoral immunity via enhanced survival, reduced apoptosis, and enhancement of activation, proliferation, class switching, and immunoglobulin (Ig) production.[67–69] These effects are best observed when B cells are additionally stimulated via the B cell receptors (BCR), CD40, or TLRs, with IFN-α being able to enhance TLR7 sensitivity of B cells by selectively upregulating TLR7 expression.[70,71] IFN-α-induced enhancement has been shown to be beneficial for generation of neutralizing antibodies, especially during viral infection.[72] In contrast, excessive production of Igs against DNA complexes in patients with systemic lupus erythematosus (SLE) is in part the result of an undesired IFN-α effect on B cells.[73] However, unlike IFN-α, the effects of IFN-λ on B cells have not been investigated in detail. Various studies have suggested that B cells express the IFN-λ receptor,[74] however, the responsiveness of B cells to IFN-λ is not well-described. Multiple myeloma B cells have been shown to proliferate in response to IFN-λ1,[75] and other studies have suggested a type I and II IFN independent mechanism of TLR7 upregulation in B cells.[76]

NK cells play an important role in the innate immune response to viral infection, specifically in their ability to recognize and respond to virus-infected cells by excretion of cytotoxic factors and cytokines (e.g. IFN-γ) that act as a first response to control the infection and activate the subsequent adaptive immune responses. The engagement of specific surface receptors on NK cells by soluble

factors, such as IL-12 and IL-18, results in cellular activation and the triggering of effector activities.[77,78] IFN-α has also been described as a potent promotor of the function and survival of NK cells, specifically in the setting of NK cell interaction with IFN-producing activated pDCs leading to enhanced activation and cytolytic activity, and thereby promoting antiviral immunity.[79–81] Additionally, during both influenza and vaccinia virus infections, the direct activation of NK cells by IFN-α is required for the expression of cytolytic mediators and production of IFN-γ.[82,83] Indirect effects of IFN-α on NK cells have also been described, mediated through DCs in an IL-15-dependent manner, in TLR-stimulated mice and during MCMV infection.[84,85] In contrast to the well-defined effects of type I IFNs on NK cells, reports about the effects of type III IFNs have been scarce and controversial. Adoptive transfer experiments in mice with IFN-λR1-deficient NK cells do suggest that a direct IFN-λ engagement is necessary for optimal production of IFN-γ and anti-tumor activity.[86] However, only a single study in humans described IFN-λ as being able to partially inhibit NK-cell-derived IFN-γ production upon IL-12/15 stimulation.[87,88] However, an effect of IFN-λ on NK cells could not be reproduced in an ensuing response to this study and was later disproved by the original study authors.[89,90]

NK CELLS IN VIRAL INFECTIONS

NK cells represent one of the first lines of defense of the innate immune response to viral infections. Activated NK cells respond to stress signals of virus-infected cells via two types of mechanisms; direct mechanisms, including the release and expression of cytotoxic mediators, such as granzymes, perforins, and tumor necrosis factor (TNF)-related apoptosis-induced ligand (TRAIL), that result in lysis of the infected cells, and indirect mechanisms that involve the release of cytokines, such as IFN-γ and TNF, and result in the triggering of antiviral responses that inhibit viral replication. These stress-induced signals include surface markers, such as altered MHC expression and ligands to regulatory receptors, as well as soluble factors, of which IL-12, IL-15, IL-18, and IFN-α/β are well described and known promotors of NK-cell effector activity.[77,78,91]

NK cell activity is strictly governed by a balance of activating and inhibitory receptors on the cell surface. Some of the major classifications of these regulatory receptors include C-type lectin-like receptors (NKG2A, NKG2C, NKG2D), natural cytotoxicity receptors (NKp30, NKp44 and NKp46), and killer cell immunoglobulin-like receptor (KIRs). During viral infection the balance shifts from inhibition to activation after a critical threshold of activation signals exceeds those of inhibition.[91] Additionally, the intensity of expression of surface glycoprotein CD56 can be used to divide NK cells into two subgroups; the less developmentally mature $CD56^{bright}$ subset, that primarily produces cytokines, and the more

developmentally mature CD56^{dim} subset that predominantly mediates cytotoxic activity. Differentiation of NK cells from CD56^{bright} to CD56^{dim} cells has also been described and shown to contribute to functional and phenotypic heterogeneity observed during the life span of NK cells. Many details of this differentiation process remain undefined, but the dynamics and outcome are likely affected by viral infections. During the differentiation process, NK cells diminish CD56 expression as well as lose expression of NKG2A, and subsequently acquire KIR and CD57 expression, ultimately resulting in a more terminally differentiated phenotype, that display reduced IFN-γ producing and proliferative capacities.[92,93]

In recent years the classic division of labor between the innate and adaptive immune systems has been challenged, with reports describing developmental and functional qualities for NK cells that are commonly associated with adaptive immune cells. Of these features, the ability of NK cells to acquire functional immunological memory and undergo continuous differentiation resulting in the formation of specific NK cell repertoires has been highlighted in viral infection settings.[94,95] In humans, a long-lived memory-like NK cell population have been described at higher frequencies in cytomegalovirus (CMV)-seropositive individuals than seronegative individuals.[96,97] This specialized population of NK cells was found to express high levels of the of C-type lectin NKG2C, in combination with maturation marker CD57. A memory phenotype for NK cells has also been suggested in *Mycobacterium tuberculosis* and hantavirus infection,[98,99] and associations of NK cell maturation and memory markers have been observed in chronic viral hepatitis patients, but only when also CMV-seropositive.[97]

AIMS AND OUTLINE OF THIS THESIS

The therapeutic use of IFNs in both HBV and HCV infection has diminished since the introduction of highly effective DAAs. However, IFNs are still potent immunomodulators that, in combination with NK cells, represent two pillars of the innate immune system in the response to pathogens and the resolution of infections. Therefore, the primary aim of the work presented in this thesis was to understand the function, and dysfunction, of these specific aspects of the innate immune system in the resolution or persistence of viral hepatitis infection.

The association of polymorphisms within the IFN-λ gene locus and spontaneous as well as therapy-induced clearance of HCV infection is still poorly understood, but suggests an important role for IFN-λ in the triggering of an effective antiviral response. Therefore, the first logical step to determine the importance of IFN-λ in viral hepatitis was to investigate the endogenous levels in the serum of chronically infected patients and how these associated with patient IFN-λ polymorphism genotypes as well as disease state/outcome (**Chapter 2).**

In addition to the role of endogenously produced IFN-λ in the setting of chronic viral hepatitis, much of the underlying basic biology of IFN-λ still remains unexplained. The antiviral aspects of this class of cytokines have been well established, with many similarities described to type I IFNs. However, this then raises the question what is the evolutionary benefit of having two classes of IFNs if their immunological role is truly redundant? Therefore, further understanding is still needed about the few distinctions described for type III IFNs, specifically the IFN-λ receptor and its expression on immune cells as well as the associated responses after triggering. With studies reporting the expression of the IFN-λ receptor on B cells but limited information available on the actual effects of IFN-λ,[74–76] we decided to investigate in detail the role of IFN-λ on the regulation and modulation of human B cell function and how this compared to that of IFN-α **(Chapter 3).**

NK cells represent another compartment of lymphocytes with poorly described response to type III IFNs, and conflicting results have been reported for both the expression of the IFN-λ receptor as well as the direct effects of IFN-λ on human NK cells.[87–90] Monocyte-derived macrophages, on the other hand, have been both described to express the IFN-λ receptor as well produce enhanced levels of IL-12p40 upon engagement of this receptor, specifically in combination with activation via TLRs.[43] This is of particular interest in hepatitis infection as TLR-activated liver-resident macrophages (Kupffer cells) have been shown to exert effector function in NK cells, mediated through cytokine induced activation and production of IFN-γ.[100] Therefore, to better understand the biological role of IFN-λ, we investigated the effects of IFN-λ on NK cells in both a direct and indirect capacity through its modulation of TLR-activated macrophages **(Chapter 4)**. Similarly, other studies have investigated the effects of IFN-λ in modulating the function of TLR-activated fibroblasts, specifically IL-6 and IL-8 production, in the setting of rheumatoid arthritis (RA) synovial inflammation. Our comments on these studies and the importance of IFN-λ in the context of inflammatory diseases, such as RA, can be found in **Chapter 5**.

As previously discussed, chronic HBV infection is characterized by disease episodes with differentiating viral loads and/or markers of liver damage (e.g. ALT). A previous systems biology study of the peripheral blood transcriptomes in the distinct chronic HBV clinical phases reported an upregulation of cytotoxicity/NK cell activity-related genes clustered in the IA and ENEG phases, suggesting a link between NK cell activity and the elevated ALT observed in these phases.[101] We therefore chose to investigate the role of differential NK cell functionalities in contributing to the distinct features observed during the HBV clinical phases, including the fluctuations in liver damage markers and HBV replication **(Chapter 6)**.

The high rates of spontaneous clearance (50 %-80 %) observed in patients that have previously resolved an acute HCV infection strongly suggest that immunological memory, and consequently immunity, acquired during the primary infection may play a role in the protection against HCV reinfection.[102,103] Recent studies have

described developmental and functional qualities for NK cells that are commonly associated with immunological memory, including the continuous differentiation and formation of specific NK cell repertoires in viral infection settings.[94–97,99] With limited information available on the development and persistence of memory-like NK cells during and after HCV infection, we chose to characterize and compare the NK cell compartments of individuals who either resolved an acute HCV infection and individuals that developed a chronic infection **(Chapter 7)**.

A summary of the work presented in this thesis, as well as the importance and implications of these studies as a whole are discussed in **Chapter 8**.

REFERENCES

1. World Health Organization (WHO). Hepatitis. (2016). Available at: http://www.who.int/hepatitis/en/.
2. World Health Organization (WHO). Hepatitis B Fact Sheet. (2016). Available at: http://www.who.int/mediacentre/factsheets/fs204/en/.
3. World Health Organization (WHO). Hepatitis C Fact Sheet. (2016). Available at: http//www.who.int/mediacentre/factsheets/fs164/en/.
4. Guidotti, L. G. & Chisari, F. V. Immunobiology and pathogenesis of viral hepatitis. *Annu. Rev. Pathol.* **1,** 23–61 (2006).
5. Di Bisceglie, A. M. Natural history of hepatitis C: its impact on clinical management. *Hepatology* **31,** 1014–8 (2000).
6. Micallef, J. M., Kaldor, J. M. & Dore, G. J. Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. *J. Viral Hepat.* **13,** 34–41 (2006).
7. GBD 2013 Mortality and Causes of Death Collaborators. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet (London, England)* **385,** 117–71 (2015).
8. Kim, W. R. *et al.* OPTN/SRTR 2013 Annual Data Report: liver. *Am. J. Transplant* **15 Suppl 2,** 1–28 (2015).
9. Shin, E.-C., Sung, P. S. & Park, S.-H. Immune responses and immunopathology in acute and chronic viral hepatitis. *Nat. Rev. Immunol.* **16,** 509–523 (2016).
10. Seeger, C. & Mason, W. S. Molecular biology of hepatitis B virus infection. *Virology* **479–480,** 672–686 (2015).
11. European Association For The Study Of The Liver. EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. *J. Hepatol.* **57,** 167–85 (2012).
12. Ashfaq, U. A., Javed, T., Rehman, S., Nawaz, Z. & Riazuddin, S. An overview of HCV molecular biology, replication and immune responses. *Virol. J.* **8,** 161 (2011).
13. Bartenschlager, R., Cosset, F.-L. & Lohmann, V. Hepatitis C virus replication cycle. *J. Hepatol.* **53,** 583–585 (2010).
14. Fried, M. W. *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N. Engl. J. Med.* **347,** 975–82 (2002).
15. Manns, M. P. *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet (London, England)* **358,** 958–65 (2001).
16. Kowdley, K. V *et al.* Ledipasvir and sofosbuvir for 8 or 12 weeks for chronic HCV without cirrhosis. *N. Engl. J. Med.* **370,** 1879–88 (2014).
17. Afdhal, N. *et al.* Ledipasvir and sofosbuvir for previously treated HCV genotype 1 infection. *N. Engl. J. Med.* **370,** 1483–93 (2014).
18. Afdhal, N. *et al.* Ledipasvir and sofosbuvir for untreated HCV genotype 1 infection. *N. Engl. J. Med.* **370,** 1889–98 (2014).
19. European Association for the Study of the Liver. Electronic address: easloffice@easloffice.eu. EASL Recommendations on Treatment of Hepatitis C 2016. *J. Hepatol.* (2016). doi:10.1016/j.jhep.2016.09.001

20. Akira, S., Takeda, K. & Kaisho, T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* **2,** 675–80 (2001).
21. Janeway, C. A. & Medzhitov, R. Innate immune recognition. *Annu. Rev. Immunol.* **20,** 197–216 (2002).
22. Medzhitov, R. & Janeway, C. A. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* **91,** 295–8 (1997).
23. Schoggins, J. W. *et al.* A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature* **472,** 481–5 (2011).
24. Li, K. *et al.* Activation of chemokine and inflammatory cytokine response in hepatitis C virus-infected hepatocytes depends on Toll-like receptor 3 sensing of hepatitis C virus double-stranded RNA intermediates. *Hepatology* **55,** 666–75 (2012).
25. Wieland, S., Thimme, R., Purcell, R. H. & Chisari, F. V. Genomic analysis of the host response to hepatitis B virus infection. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 6669–74 (2004).
26. Wieland, S. F. & Chisari, F. V. Stealth and cunning: hepatitis B and hepatitis C viruses. *J. Virol.* **79,** 9369–80 (2005).
27. Pestka, S., Krause, C. D. & Walter, M. R. Interferons, interferon-like cytokines, and their receptors. *Immunol. Rev.* **202,** 8–32 (2004).
28. Cella, M. *et al.* Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat. Med.* **5,** 919–23 (1999).
29. Siegal, F. P. *et al.* The nature of the principal type 1 interferon-producing cells in human blood. *Science* **284,** 1835–7 (1999).
30. Schoenborn, J. R. & Wilson, C. B. Regulation of interferon-gamma during innate and adaptive immune responses. *Adv. Immunol.* **96,** 41–101 (2007).
31. Prokunina-Olsson, L. *et al.* A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nat. Genet.* **45,** 164–71 (2013).
32. Ge, D. *et al.* Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* **461,** 399–401 (2009).
33. Thomas, D. L. *et al.* Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* **461,** 798–801 (2009).
34. Tanaka, Y. *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat. Genet.* **41,** 1105–9 (2009).
35. Suppiah, V. *et al.* IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat. Genet.* **41,** 1100–4 (2009).
36. Park, H. *et al.* IL-29 is the dominant type III interferon produced by hepatocytes during acute hepatitis C virus infection. *Hepatology* **56,** 2060–70 (2012).
37. Yin, Z. *et al.* Type III IFNs are produced by and stimulate human plasmacytoid dendritic cells. *J. Immunol.* **189,** 2735–45 (2012).
38. Lauterbach, H. *et al.* Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. *J. Exp. Med.* **207,** 2703–17 (2010).
39. Sheppard, P. *et al.* IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat. Immunol.* **4,** 63–8 (2003).
40. Kotenko, S. V *et al.* IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat. Immunol.* **4,** 69–77 (2003).

41. Ivashkiv, L. B. & Donlin, L. T. Regulation of type I interferon responses. *Nat. Rev. Immunol.* **14,** 36–49 (2014).
42. Ank, N. *et al.* An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. *J. Immunol.* **180,** 2474–85 (2008).
43. Liu, B.-S., Janssen, H. L. A. & Boonstra, A. IL-29 and IFN-α differ in their ability to modulate IL-12 production by TLR-activated human macrophages and exhibit differential regulation of the IFNγ receptor expression. *Blood* **117,** 2385–95 (2011).
44. Xu, L. *et al.* IL-29 enhances Toll-like receptor-mediated IL-6 and IL-8 production by the synovial fibroblasts from rheumatoid arthritis patients. *Arthritis Res. Ther.* **15,** R170 (2013).
45. Megjugorac, N. J., Gallagher, G. E. & Gallagher, G. Modulation of human plasmacytoid DC function by IFN-lambda1 (IL-29). *J. Leukoc. Biol.* **86,** 1359–63 (2009).
46. Makowska, Z., Duong, F. H. T., Trincucci, G., Tough, D. F. & Heim, M. H. Interferon-β and interferon-λ signaling is not affected by interferon-induced refractoriness to interferon-α in vivo. *Hepatology* **53,** 1154–63 (2011).
47. François-Newton, V. *et al.* USP18-based negative feedback control is induced by type I and type III interferons and specifically inactivates interferon α response. *PLoS One* **6,** e22200 (2011).
48. Olagnier, D. & Hiscott, J. Type I and type III interferon-induced immune response: it's a matter of kinetics and magnitude. *Hepatology* **59,** 1225–8 (2014).
49. Bolen, C. R., Ding, S., Robek, M. D. & Kleinstein, S. H. Dynamic expression profiling of type I and type III interferon-stimulated hepatocytes reveals a stable hierarchy of gene expression. *Hepatology* **59,** 1262–72 (2014).
50. Jilg, N. *et al.* Kinetic differences in the induction of interferon stimulated genes by interferon-α and interleukin 28B are altered by infection with hepatitis C virus. *Hepatology* **59,** 1250–61 (2014).
51. Horner, S. M. & Gale, M. Regulation of hepatic innate immunity by hepatitis C virus. *Nat. Med.* **19,** 879–88 (2013).
52. Chen, J. *et al.* Hepatitis B virus polymerase impairs interferon-α-induced STA T activation through inhibition of importin-α5 and protein kinase C-δ. *Hepatology* **57,** 470–82 (2013).
53. Liu, Y. *et al.* Hepatitis B virus polymerase disrupts K63-linked ubiquitination of STING to block innate cytosolic DNA-sensing pathways. *J. Virol.* **89,** 2287–300 (2015).
54. Wang, H. & Ryu, W.-S. Hepatitis B virus polymerase blocks pattern recognition receptor signaling via interaction with DDX3: implications for immune evasion. *PLoS Pathog.* **6,** e1000986 (2010).
55. Wei, C. *et al.* The hepatitis B virus X protein disrupts innate immunity by downregulating mitochondrial antiviral signaling protein. *J. Immunol.* **185,** 1158–68 (2010).
56. Bender, S. *et al.* Activation of Type I and III Interferon Response by Mitochondrial and Peroxisomal MAVS and Inhibition by Hepatitis C Virus. *PLoS Pathog.* **11,** e1005264 (2015).
57. Li, K. *et al.* Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 2992–7 (2005).
58. Montoya, M. *et al.* Type I interferons produced by dendritic cells promote their phenotypic and functional activation. *Blood* **99,** 3263–71 (2002).

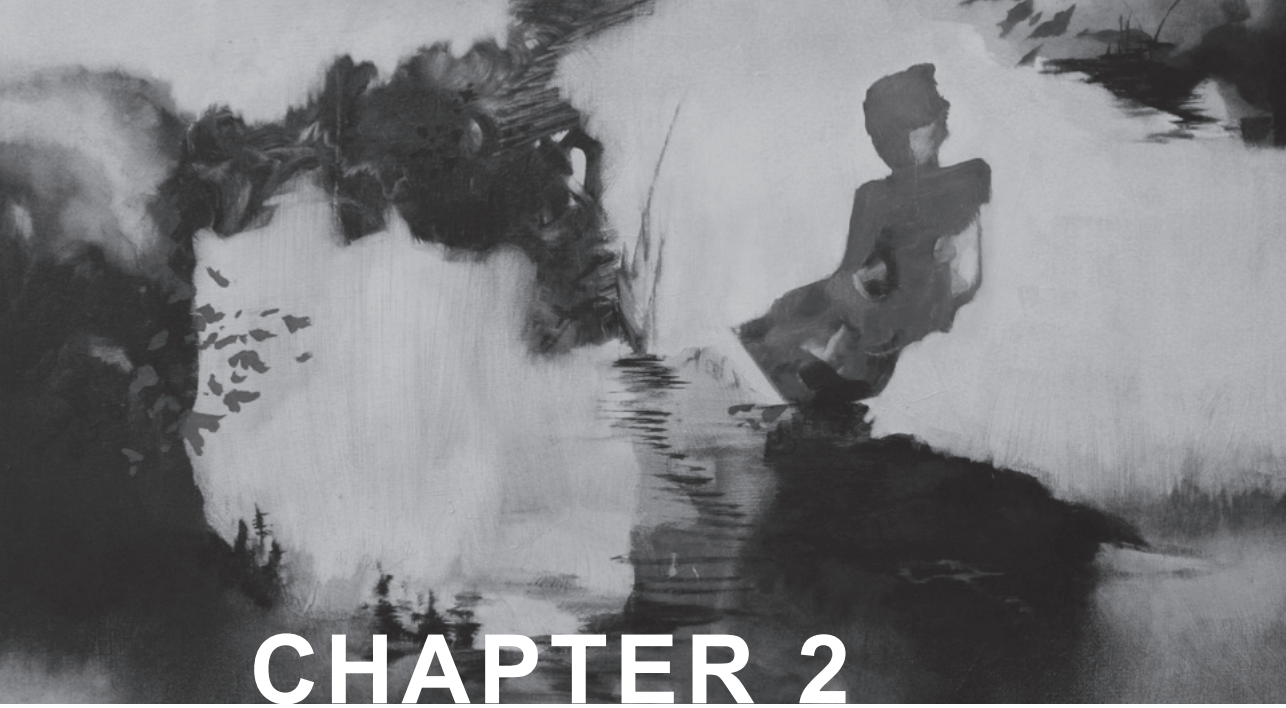
59. Ito, T. *et al.* Differential regulation of human blood dendritic cell subsets by IFNs. *J. Immunol.* **166,** 2961–9 (2001).
60. Hahm, B., Trifilo, M. J., Zuniga, E. I. & Oldstone, M. B. A. Viruses evade the immune system through type I interferon-mediated STAT2-dependent, but STAT1-independent, signaling. *Immunity* **22,** 247–57 (2005).
61. Gautier, G. *et al.* A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. *J. Exp. Med.* **201,** 1435–46 (2005).
62. Byrnes, A. A. *et al.* Type I interferons and IL-12: convergence and cross-regulation among mediators of cellular immunity. *Eur. J. Immunol.* **31,** 2026–34 (2001).
63. McRae, B. L., Semnani, R. T., Hayes, M. P. & van Seventer, G. A. Type I IFNs inhibit human dendritic cell IL-12 production and Th1 cell development. *J. Immunol.* **160,** 4298–304 (1998).
64. Trinchieri, G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* **3,** 133–46 (2003).
65. Boonstra, A. *et al.* Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. *J. Immunol.* **177,** 7551–8 (2006).
66. Mosser, D. M. & Edwards, J. P. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* **8,** 958–69 (2008).
67. Swanson, C. L. *et al.* Type I IFN enhances follicular B cell contribution to the T cell-independent antibody response. *J. Exp. Med.* **207,** 1485–500 (2010).
68. Le Bon, A. *et al.* Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. *J. Immunol.* **176,** 2074–8 (2006).
69. Le Bon, A. *et al.* Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. *Immunity* **14,** 461–70 (2001).
70. Bekeredjian-Ding, I. B. *et al.* Plasmacytoid dendritic cells control TLR7 sensitivity of naive B cells via type I IFN. *J. Immunol.* **174,** 4043–50 (2005).
71. Ruprecht, C. R. & Lanzavecchia, A. Toll-like receptor stimulation as a third signal required for activation of human naive B cells. *Eur. J. Immunol.* **36,** 810–6 (2006).
72. Coro, E. S., Chang, W. L. W. & Baumgarth, N. Type I IFN receptor signals directly stimulate local B cells early following influenza virus infection. *J. Immunol.* **176,** 4343–51 (2006).
73. Crow, M. K. & Kirou, K. A. Interferon-alpha in systemic lupus erythematosus. *Curr. Opin. Rheumatol.* **16,** 541–7 (2004).
74. Witte, K. *et al.* Despite IFN-lambda receptor expression, blood immune cells, but not keratinocytes or melanocytes, have an impaired response to type III interferons: implications for therapeutic applications of these cytokines. *Genes Immun.* **10,** 702–14 (2009).
75. Novak, A. J. *et al.* A role for IFN-lambda1 in multiple myeloma B cell growth. *Leukemia* **22,** 2240–6 (2008).
76. Sinha, S., Guo, Y., Thet, S. & Yuan, D. IFN type I and type II independent enhancement of B cell TLR7 expression by natural killer cells. *J. Leukoc. Biol.* **92,** 713–22 (2012).
77. Lanier, L. L. NK cell recognition. *Annu. Rev. Immunol.* **23,** 225–74 (2005).
78. Vivier, E. *et al.* Innate or adaptive immunity? The example of natural killer cells. *Science* **331,** 44–9 (2011).

79. Gerosa, F. *et al.* The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. *J. Immunol.* **174,** 727–34 (2005).
80. Trinchieri, G., Santoli, D., Dee, R. R. & Knowles, B. B. Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Identification of the anti-viral activity as interferon and characterization of the human effector lymphocyte subpopulation. *J. Exp. Med.* **147,** 1299–1313 (1978).
81. Trinchieri, G. & Santoli, D. Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis. *J. Exp. Med.* **147,** 1314–33 (1978).
82. Martinez, J., Huang, X. & Yang, Y. Direct action of type I IFN on NK cells is required for their activation in response to vaccinia viral infection in vivo. *J. Immunol.* **180,** 1592–7 (2008).
83. Hwang, I. *et al.* Activation mechanisms of natural killer cells during influenza virus infection. *PLoS One* **7,** e51858 (2012).
84. Baranek, T. *et al.* Differential responses of immune cells to type I interferon contribute to host resistance to viral infection. *Cell Host Microbe* **12,** 571–84 (2012).
85. Lucas, M., Schachterle, W., Oberle, K., Aichele, P. & Diefenbach, A. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity* **26,** 503–17 (2007).
86. Souza-Fonseca-Guimaraes, F. *et al.* NK cells require IL-28R for optimal in vivo activity. *Proc. Natl. Acad. Sci. U. S. A.* **112,** E2376-84 (2015).
87. Gardiner, C. M., Morrison, M. H. & Dring, M. M. Reply to Kramer et al.: Human natural killer (NK) cell inhibition by IL28A. *Proc. Natl. Acad. Sci.* **108,** E521–E522 (2011).
88. Dring, M. M. *et al.* Innate immune genes synergize to predict increased risk of chronic disease in hepatitis C virus infection. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 5736–41 (2011).
89. Morrison, M. H. *et al.* IFNL cytokines do not modulate human or murine NK cell functions. *Hum. Immunol.* **75,** 996–1000 (2014).
90. Krämer, B. *et al.* Do lambda-IFNs IL28A and IL28B act on human natural killer cells? *Proc. Natl. Acad. Sci. U. S. A.* **108,** E519-20–2 (2011).
91. Long, E. O., Kim, H. S., Liu, D., Peterson, M. E. & Rajagopalan, S. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu. Rev. Immunol.* **31,** 227–58 (2013).
92. Björkström, N. K. *et al.* Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood* **116,** 3853–64 (2010).
93. Béziat, V., Descours, B., Parizot, C., Debré, P. & Vieillard, V. NK cell terminal differentiation: correlated stepwise decrease of NKG2A and acquisition of KIRs. *PLoS One* **5,** e11966 (2010).
94. O'Sullivan, T. E., Sun, J. C. & Lanier, L. L. Natural Killer Cell Memory. *Immunity* **43,** 634–45 (2015).
95. Sun, J. C., Lopez-Verges, S., Kim, C. C., DeRisi, J. L. & Lanier, L. L. NK cells and immune "memory". *J. Immunol.* **186,** 1891–7 (2011).

96. Lopez-Vergès, S. *et al.* Expansion of a unique CD57^{+}NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 14725–32 (2011).
97. Béziat, V. *et al.* CMV drives clonal expansion of NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients. *Eur. J. Immunol.* **42,** 447–57 (2012).
98. Fu, X., Yang, B., Lao, S., Fan, Y. & Wu, C. Human memory-like NK cells migrating to tuberculous pleural fluid via IP-10/CXCR3 and SDF-1/CXCR4 axis produce IFN-γ in response to Bacille Calmette Guerin. *Clin. Immunol.* **148,** 113–23 (2013).
99. Björkström, N. K. *et al.* Rapid expansion and long-term persistence of elevated NK cell numbers in humans infected with hantavirus. *J. Exp. Med.* **208,** 13–21 (2011).
100. Tu, Z. *et al.* TLR-dependent cross talk between human Kupffer cells and NK cells. *J. Exp. Med.* **205,** 233–44 (2008).
101. Vanwolleghem, T. *et al.* Re-evaluation of hepatitis B virus clinical phases by systems biology identifies unappreciated roles for the innate immune response and B cells. *Hepatology* **62,** 87–100 (2015).
102. Sacks-Davis, R. *et al.* Hepatitis C Virus Reinfection and Spontaneous Clearance of Reinfection—the InC3 Study. *J. Infect. Dis.* **212,** 1407–19 (2015).
103. Osburn, W. O. *et al.* Spontaneous control of primary hepatitis C virus infection and immunity against persistent reinfection. *Gastroenterology* **138,** 315–24 (2010).

PART 1

SYSTEMIC LEVELS AND IMMUNOLOGICAL REGULATION OF TYPE III IFNS IN VIRAL HEPATITIS INFECTION



CHAPTER 2

ENDOGENOUS IFN-Λ IN VIRAL HEPATITIS PATIENTS

Rik A. de Groen[1], Fiona Mcphee[2], Jacques Friborg[2], Harry L.A. Janssen[1,3], and André Boonstra[1]

[1] Department of Gastroenterology and Hepatology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[2] Research and Development, Bristol-Myers Squibb, Wallingford, CT, USA
[3] Liver Clinic University Health Network, Division of Gastroenterology, University of Toronto, Canada

J INTERFERON CYTOKINE RES. 2014 JUL;34(7):552-6.

ABSTRACT

Besides type I interferons, type III interferons, including IFN-λ1 (IL-29), possess potent antiviral activity. In patients infected with the hepatitis C virus (HCV), it has been demonstrated that viral clearance is associated with genetic variation near the IFN-λ3 (IL-28B) gene. The rapid influx of research being conducted on this family of cytokines has led to several inconsistencies and controversies, including the possible correlation of serum cytokine levels with disease in chronic viral hepatitis patients. In a detailed study, well-characterized cohorts of patients with HBV and HCV were evaluated with 3 different immunoassays, and no differences in the levels of serum IFN-λ were observed between patient groups, disease stages or clinical parameters.

INTRODUCTION

Hepatitis C virus (HCV) and hepatitis B virus (HBV) infection are two of the leading causes of chronic liver disease and inflammation worldwide. More than 500 million people are persistently infected with HBV and/or HCV and are at a serious risk of developing chronic liver disease, cirrhosis, and hepatocellular carcinoma (Lavanchy 2008).

Endogenous interferons (IFN), specifically IFN-α, play a pivotal role in host defense against viral infection, and has also been one of the cornerstones for the treatment of chronic HCV and HBV infection over the past 20 years. Recently a distinct class of IFNs, the family of IFN-λ, has become the topic of intense investigation in the field of viral hepatitis originating with the observation that single nucleotide polymorphisms (SNPs) near the gene encoding for IFN-λ3 displayed an exceptionally strong association with spontaneous as well as therapy-induced clearance of HCV (Ge and others 2009; Suppiah and others 2009; Tanaka and others 2009; Thomas and others 2009). IFN-λ1 (also known as interleukin-29 or IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B) are known to possess potent antiviral activity via mechanisms similar to those of IFN-α despite triggering a unique IFN-λ receptor pair (Doyle and others 2006; Dumoutier and others 2004; Zhou and others 2007). The IFN-α receptor is composed of 2 unique receptor chains, IFN-αR1 and IFN-αR2, whereas the IFN-λ receptor is comprised of IFN-λ receptor 1 (IFN-λR1, also known as IL-28RA) and IL-10 receptor 2 (IL-10R2). IFN-αR1, IFN-αR2, and IL-10R2 are ubiquitously expressed, while the expression of IFN-λR1 appears to be limited to plasmacytoid DC, B cells, epithelial cells, hepatocytes, and macrophages (Dumoutier and others 2004; Liu and others 2011). The more restricted expression of IFN-λR1 could potentially make IFN-λ a more specific antiviral therapeutic, and explain the reduced incidence of side-effects reported during antiviral therapy of pegylated-IFN-λ1 as compared to pegylated-IFN-α treatment (Muir and others 2010). Current clinical trials are further examining benefits of using pegylated-IFN-λ1 as an alternative to conventional IFN-α based treatment of chronic HCV infection.

The association of the IFN-λ3 gene polymorphisms with spontaneous clearance of acute HCV infection suggests an important role for IFN-λ in triggering an effective antiviral response in infected individuals. The first logical step to determine the importance of endogenous IFN-λ in chronic HCV is to investigate the levels in the blood of chronically infected HCV patients. However, the rapid influx of research being conducted on this family of cytokines have led to several inconsistencies and controversies. This includes incongruities in the levels of IFN-λ measured in the sera of HCV patients and healthy individuals as well as cell populations that express IFN-λR1 (Dring and others 2011; Kramer and others 2011). These conflicting results show that IFN-λ levels in the serum of healthy controls are higher than those of chronically infected HCV patients (Langhans

and others 2011), and conversely that HCV patient IFN-λ serum levels are elevated compared to those of healthy individuals (Diegelmann and others 2010; Dolganiuc and others 2012). To resolve these discrepancies and understand the role of IFN-λ in viral clearance we performed a detailed cross-sectional analysis of IFN-λ sera levels in a large cohort of HCV infected, HBV infected, and healthy individuals. Furthermore, to confirm and validate our findings, serum IFN-λ concentrations were determined by 3 different immunoassays.

MATERIALS AND METHODS

Patients

Sera were collected from the blood of 124 chronically infected HCV patients, 22 resolved HCV patients, 93 chronically infected HBV patients, 20 acutely infected HBV patients, and 25 chronically infected HBV patients currently receiving

Table 1: Patient Characteristics

		Average Age (years)	Sex (% male)	ALT (IU/ml)	HCV/HBV viral load (IU/ml)	HBeAg (% positive)	IFN-λ3 rs12989860 SNP (%; TT, CT, CC)
Healthy	Control (n = 20)	34 (25–56)	46	-	-	-	-
HCV	Chronic (n = 47)	54 (34–47)	72	80 (18–851)	$2.7x10^6$ ($1.4x10^1$–$1.3x10^7$)	-	23; 45; 32
	"High ALT" (n = 37)	54 (29–96)	73	243 (160–013)	$1.1x10^7$ ($6.9x10^5$–$5.3x10^7$)	-	-
	"Low ALT" (n = 40)	54 (27–76)	55	29 (11–19)	$3.3x10^6$ ($3.0x10^5$–$5.3x10^7$)	-	-
	Resolved (n = 22)	53 (26–67)	77	30 (11–17)	< 15	-	52; 24; 24
HBV	Chronic (n = 25)	37 (19–93)	44	48 (15–567)	$4.5x10^8$ ($2.0x10^1$–$1.9x10^9$)	48	-
	Immune-active (n = 23)	35 (20–08)	73	96 (24–453)	$5.8x10^{\wedge 8}$ ($2.0x10^1$–$1.9x10^9$)	65	-
	Immune-tolerant (n = 15)	33 (21–19)	33	21 (14–48)	$6.0x10^{\wedge 8}$ ($1.1x10^2$–$2.76x10^9$)	100	-
	Inactive carriers (n = 30)	38 (17–72)	40	24 (13–37)	$2.3x10^4$ ($2.0x10^1$–$1.3x10^5$)	3	-
	Therapy (n = 25)	45 (27–78)	64	37 (12–21)	$3.8x10^4$ ($2.0x10^1$–$1.9x10^5$)	19	-
	Acute (n = 20)	-	-	-	-	-	-

nucleot(s)ide analog therapy, who visited the outpatient clinic. The characteristics of the enrolled subjects are listed in Table 1. As presented in Table 1, 77 out of the 124 chronic HCV patients were selected from our bio-bank on the basis of their ALT values, being either lower than 40 IU/l or higher than 160 IU/l. After collection, the sera were immediately aliquoted and stored at -80°C. Serum HCV-RNA levels were determined by quantitative PCR (Cobas® Ampliprep/ Cobas® TaqMan® HCV test (limit of detection < 15 IU/mL, Roche Diagnostics, the Netherlands), and serum HBV DNA levels were determined by standard clinical diagnostics of the Erasmus MC, Rotterdam. In addition, sera from blood of 20 healthy individuals were included in the study. The institutional ethical review board of the Erasmus MC, Rotterdam approved the clinical protocols, and written informed consent was obtained from all individuals prior to their inclusion. The study was conducted according to the principles expressed in the Declaration of Helsinki.

IL-28B genotype determination

The IL-28B rs12979860 gene polymorphisms were determined using competitive allele-specific PCR (KASP; KBioscience Hoddesdon, UK).

Immunoassay for detection of IFN-λ1 and IFN-λ2/3 in serum

IFN-λ concentrations in the sera of individuals were determined using sandwich ELISA specific for IFN-λ1 (Human IFN lambda 1 ELISA Ready-Set-Go, eBioscience) with a detection limit of 8 pg/ml and a validation range between 8 and 1000 pg/ml. These results were then validated using ELISA reagents from other commercial/non-commercial sources (Verikine-DIY™ Human Interferon Lambda ELISA, PBL Interferon Source, and Human IFN-λ1 ELISA, Bristol-Myers Squibb (Murine Anti-IFN-λ1 Capture Antibody E10137, Murine Anti- IFN-λ1 Biotinylated Bio-E7900). Due to the fact that IFN-λ2 and IFN-λ3 are 96% homologous, a single IFN-λ2/3 sandwich ELISA (Verikine-DIY™ Human Interferon Lambda 2/3 ELISA, PBL Interferon Source) was used to measure the combined cytokine levels (detection limit of 125 pg/ml and a validation range between 125 and 8000 pg/ml).

STATISTICAL ANALYSIS

Data is expressed as mean values ± standard error of the mean (SEM) unless indicated otherwise. Data was analyzed with Prism 5.0 software (GraphPad) using the Mann-Whitney U test to compare variables between 2 independent groups and the Spearman rank correlation coefficient test for nonparametric correlations. In all analyses, a 2-tailed P value of less than 0.05 (confidence interval of 95%) was considered statistically significant.

Results & Discussion

To examine the role of endogenous IFN-λ in chronically infected HBV and HCV patients, we performed a cross-sectional analysis of IFN-λ1 and IFN-λ2/3 serum levels in 124 chronically infected HCV patients, 22 HCV resolved HCV patients, 93 chronically infected HBV patients, 25 chronically infected HBV patients currently receiving nucleot(s)ide analog therapy, 20 acutely infected HBV patients, and 20 healthy individuals (Table 1).

No significant differences were identified when comparing the serum IFN-λ1 levels of healthy individuals to those of chronically infected HCV ($p = 0.169$) and HCV patients with therapy-induced SVR ($p = 0.115$) or between the patient groups themselves ($p = 0.357$) (Fig. 1A). Next, we stratified the HCV patients on the basis of IFN-λ3 (IL-28B) genotype as well as various clinical parameters, after which we performed further cross-sectional analysis to determine if any of these factors were associated with IFN-λ1 levels in serum. Of the total chronic HCV patient group, 47 were genotyped for the IFN-λ3 SNP at loci rs12979860, with a C allele frequency of 45.7%. We observed no difference in IFN-λ1 levels between patients homozygous for the C allele, heterozygous patients, and those homozygous for the T allele (Fig. 1B). Serum ALT (Fig. 1C, 1D), HCV viremia, and degree of liver fibrosis were all found to not significantly correlate with IFN-λ1 levels in the sera of HCV infected individuals (data not shown). Serum IFN-λ1 levels of patients with varying ALT levels were further compared by selecting patients within 2 clinically relevant groups; those with normal ALTs ("Low ALT" ≤ 40IU/l) and those with extremely elevated ALT levels ("High ALT" > 160IU/l; 4x the norm) ($p = 0.137$). The ALT levels of these patients were checked to confirm they had remained consistent for at least 6 months prior to the date of blood collection. We found it necessary to ensure that the reagents used would not bias any results, and validated all measurements using a secondary and tertiary ELISA kit from different commercial/non-commercial sources. Although it is notable that some of the individual measurements did vary between the immunoassay kits, the overall results remained unaffected and validated that there are no differences in endogenous IFN-λ1 serum levels of chronic HCV patients as compared to healthy individuals. Additionally, the levels of serum IFN-λ2/3 were measured for patients chronically infected with HCV and healthy individuals and no differences between individual groups or correlations with clinical parameters were observed (Fig. 1E-F).

Furthermore, we examined whether patients chronically infected with HBV demonstrated enhanced levels of IFN-λ1 in serum. As shown in Fig. 2A, no differences were observed when comparing chronic HBV patients with healthy individuals or chronic HCV patients. Serum IFN-λ1 levels of chronically infected HBV patients receiving nucleot(s)ide analog therapy ($n = 25$), and acutely infected HBV patients ($n = 20$) were also measured, and showed no significant differences when compared to any of the aforementioned patient/healthy control groups

(Fig. 2A-B). The chronic HBV patients were further divided in 3 clinical groups; immune-active, immune-tolerant, and inactive carriers but again no differences in IFN-λ1 serum levels were observed (Fig. 2C). All measurements were validated using additional ELISA kits, and the results remained consistent across reagent kits used, showing no significant differences or correlations between any clinical groups.

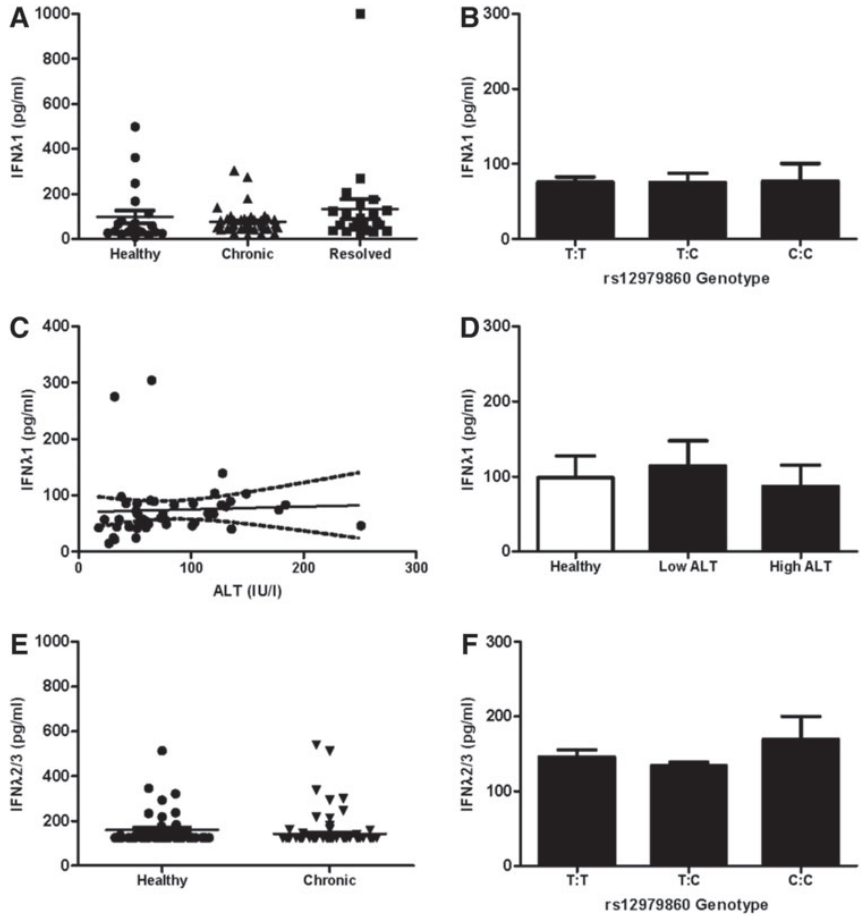


Figure 1. IFN-λ1 and IFN-λ2/3 serum levels of HCV patients do not differ from those of healthy individuals, and do not correlate with various genotypic and clinical factors.

(A) Levels of IFN-λ1 were measured from the sera of various healthy and HCV patient populations. (B) IFN-λ1 serum levels of chronic HCV patients were stratified according to their rs12979860 (IL-28B) genotype. (C) IFN-λ1 serum levels of chronic HCV patients were plotted against their corresponding ALTs. (D) A second group of patients was selected with either clinically normal ALTs ("Low ALT" ≤ 40IU/l) or those with extremely (4x the norm) elevated ALT levels ("High ALT" > 160 IU/l). (E) IFN-λ2/3 levels were measured from sera of healthy and HCV patient populations. (F) IFN-λ2/3 serum levels of chronic HCV patients were stratified according to their rs12979860 (IL-28B) genotype.

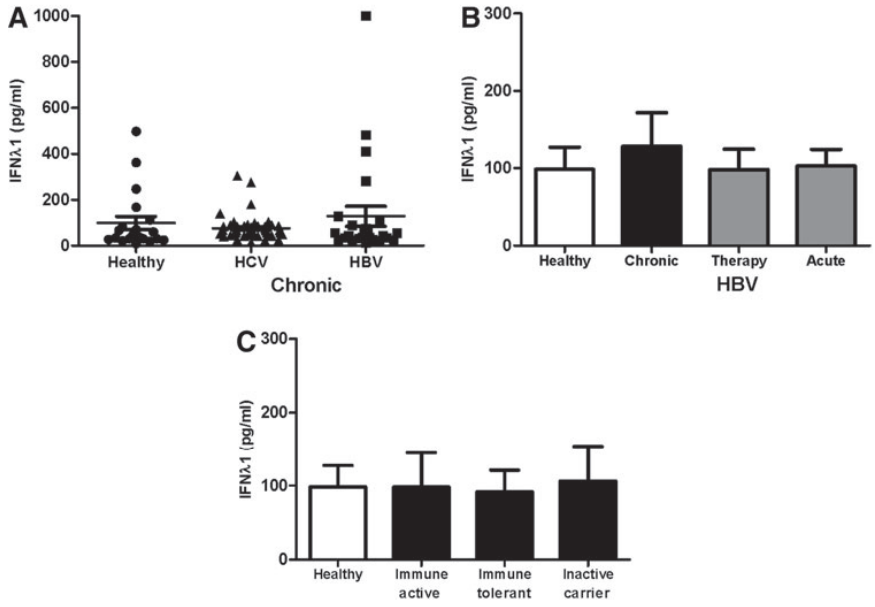


Figure 2. Endogenous IFN-λ1 levels do not differ in serum of acute and various chronic HBV patient groups.

(A) IFN-λ1 levels measured from the sera of healthy, chronic HCV, and chronic HBV patient populations. (B) IFN-λ1 serum levels of healthy controls, chronic HBV patients, chronic HBV patients receiving nucleot(s)ide analog therapy, and acute HBV patients. (C) IFN-λ1 serum levels of immune-active, immune-tolerant, and inactive-carrier chronic HBV patients.

We investigated the role of endogenous IFN-λ levels in the blood of viral hepatitis patients and observed that contrary to previous findings, no significant patterns emerged when comparing the serum IFN-λ1 or IFN-λ2/3 levels of healthy controls and patients with various genotypic and clinical factors (Diegelmann and others 2010; Dolganiuc and others 2012; Langhans and others 2011). It has also been previously suggested that decreased IFN-λ levels of chronic HCV patients may partially explain disease progression to chronicity and associations between IFN-λ3 SNPs with spontaneous and therapy induced viral clearance (Langhans and others 2011). Although our findings are similar in respect to IFN-λ2/3, we did not detect a decrease in IFN-λ1 levels in the blood of chronic HCV or HBV patients and no correlation between serum IFN-λ levels and rs12979860 genotypes, suggesting that no such link exists. Although there is a clear link between rs12979860 and various other IFN-λ3 SNPs with HCV self-clearance and probability of patient response to therapy, this is not explained by differences in endogenous IFN-λ levels. Outside of these differences observed in chronic HCV patients our results, regarding the levels and range of IFN-λ1 detected in healthy individuals and therapy or spontaneously resolved patients, resemble

those described previously described (Langhans and others 2011). To try and avoid biases created by patient cohort selection or reagents used, we validated our original findings using patients with extremes for various clinical criteria (ALT and viral load) to rule out the influence of these factors and used secondary and tertiary immunoassays from different distributers to validate our results. Additionally, when comparing the IFN-λ1 levels in the serum of healthy individuals, chronic HCV, and resolved HCV patients we observed no differences, suggesting that quantity of endogenous IFN-λ1 in blood is not significantly affected during viral hepatitis or the resolution of it. With respect to IFN-λ2 and IFN-λ3, it is notable that these cytokines are virtually identical, with a 96% amino acid homology (Sheppard and others 2003) and current reagents have difficulty measuring these individual cytokines accurately due to cross-reactivity.

Our data, which was obtained from a large cohort of well-defined patients and validated by multiple, distinct immunoassays, demonstrate that serum IFN-λ levels are not affected by infection with HCV or HBV, and not associated with specific disease stages or parameters. This study is also the first to in detail describe the IFN-λ serum levels of various groups of HBV infected patients. Our findings, however, do not exclude the possibility that local production of IFN in the liver is affected, or that the novel member the type III interferon family, IFN-λ4 (Prokunina-Olsson and others 2013) demonstrates differential expression in disease. Lastly, endogenous IFN-λ could have little effect on viral clearance, and that difference in sensitivity or response of cells determines the disease or treatment outcome. Either way, continued research on the IFN-λ family of cytokines is necessary to fully understand its immunological activity, and its possible benefits or disadvantages as a therapeutic.

ACKNOWLEDGMENTS

This study was supported by the Virgo consortium, funded by the Dutch government project number FES0908, and by the Netherlands Genomics Initiative (NGI) project number 050–060–452; and further supported by funding of Bristol-Meyers Squibb.

AUTHOR'S CONTRIBUTIONS

RdG designed research, performed research, analyzed data and wrote the manuscript; FM, JF and HLAJ designed research and wrote the paper; AB designed research, analyzed data and wrote the manuscript.

REFERENCES

Diegelmann J, Beigel F, Zitzmann K, Kaul A, Goke B, Auernhammer CJ, Bartenschlager R, Diepolder HM, Brand S. 2010. Comparative analysis of the lambda-interferons IL-28A and IL-29 regarding their transcriptome and their antiviral properties against hepatitis C virus. PLoS One 5(12):e15200.

Dolganiuc A, Kodys K, Marshall C, Saha B, Zhang S, Bala S, Szabo G. 2012. Type III interferons, IL-28 and IL-29, are increased in chronic HCV infection and induce myeloid dendritic cell-mediated FoxP3+ regulatory T cells. PLoS One 7(10):e44915.

Doyle SE, Schreckhise H, Khuu-Duong K, Henderson K, Rosler R, Storey H, Yao L, Liu H, Barahmand-pour F, Sivakumar P, Chan C, Birks C, Foster D, Clegg CH, Wietzke-Braun P, Mihm S, Klucher KM. 2006. Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. Hepatology 44(4):896-906.

Dring MM, Morrison MH, McSharry BP, Guinan KJ, Hagan R, Irish HCVRC, O'Farrelly C, Gardiner CM. 2011. Innate immune genes synergize to predict increased risk of chronic disease in hepatitis C virus infection. Proc Natl Acad Sci U S A 108(14):5736-41.

Dumoutier L, Tounsi A, Michiels T, Sommereyns C, Kotenko SV, Renauld JC. 2004. Role of the interleukin (IL)-28 receptor tyrosine residues for antiviral and antiproliferative activity of IL-29/interferon-lambda 1: similarities with type I interferon signaling. J Biol Chem 279(31):32269-74.

Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB. 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461(7262): 399-401.

Kramer B, Eisenhardt M, Glassner A, Korner C, Sauerbruch T, Spengler U, Nattermann J. 2011. Do lambda-IFNs IL28A and IL28B act on human natural killer cells? Proc Natl Acad Sci U S A 108(34):E519-20; author reply E521-2.

Langhans B, Kupfer B, Braunschweiger I, Arndt S, Schulte W, Nischalke HD, Nattermann J, Oldenburg J, Sauerbruch T, Spengler U. 2011. Interferon-lambda serum levels in hepatitis C. J Hepatol 54(5):859-65.

Lavanchy D. 2008. Chronic viral hepatitis as a public health issue in the world. Best Pract Res Clin Gastroenterol 22(6):991-1008.

Liu BS, Janssen HL, Boonstra A. 2011. IL-29 and IFNalpha differ in their ability to modulate IL-12 production by TLR-activated human macrophages and exhibit differential regulation of the IFNgamma receptor expression. Blood 117(8):2385-95.

Muir AJ, Shiffman ML, Zaman A, Yoffe B, de la Torre A, Flamm S, Gordon SC, Marotta P, Vierling JM, Lopez-Talavera JC, Byrnes-Blake K, Fontana D, Freeman J, Gray T, Hausman D, Hunder NN, Lawitz E. 2010. Phase 1b study of pegylated interferon lambda 1 with or without ribavirin in patients with chronic genotype 1 hepatitis C virus infection. Hepatology 52(3):822-32.

Prokunina-Olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, Dickensheets H, Hergott D, Porter-Gill P, Mumy A, Kohaar I, Chen S, Brand N, Tarway M, Liu L, Sheikh F, Astemborski J, Bonkovsky HL, Edlin BR, Howell CD, Morgan TR, Thomas DL, Rehermann B, Donnelly RP, O'Brien TR. 2013. A variant upstream of IFNL3 (IL28B)

creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. Nat Genet 45(2):164-71.

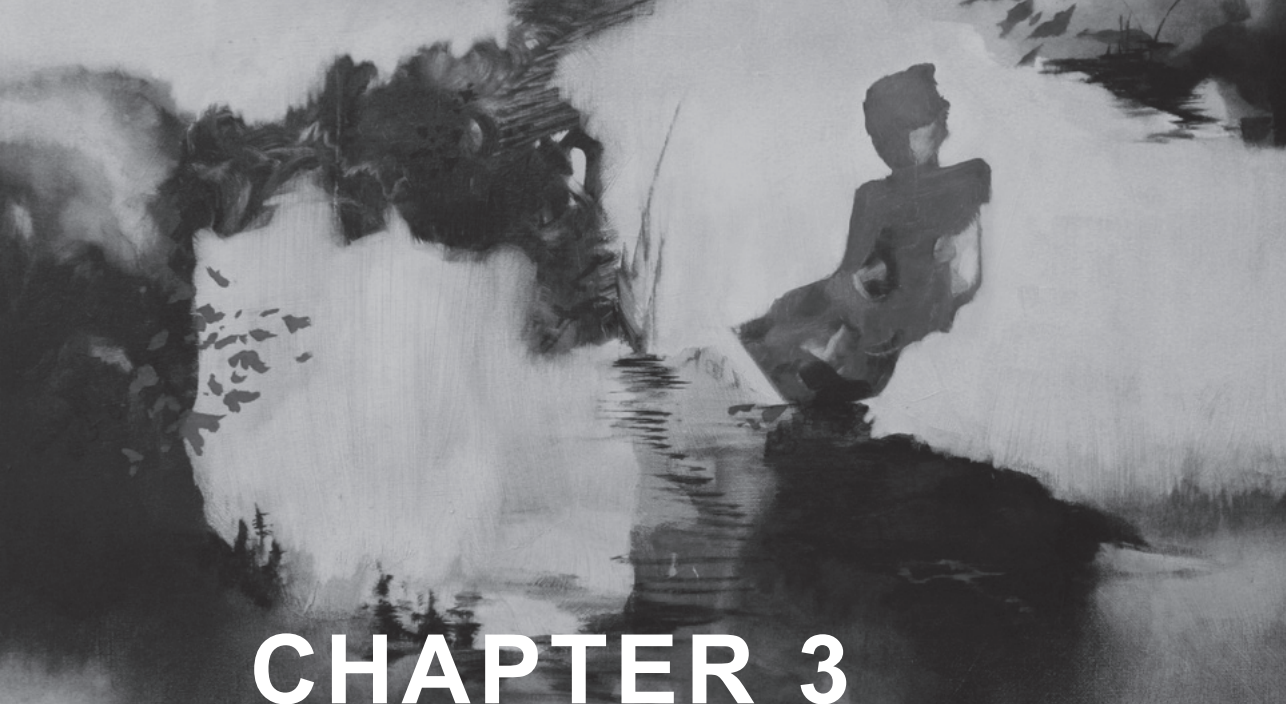
Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, Kuestner R, Garrigues U, Birks C, Roraback J, Ostrander C, Dong D, Shin J, Presnell S, Fox B, Haldeman B, Cooper E, Taft D, Gilbert T, Grant FJ, Tackett M, Krivan W, McKnight G, Clegg C, Foster D, Klucher KM. 2003. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 4(1):63-8.

Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Muller T, Bahlo M, Stewart GJ, Booth DR, George J. 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41(10):1100-4.

Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41(10):1105-9.

Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, Kidd J, Kidd K, Khakoo SI, Alexander G, Goedert JJ, Kirk GD, Donfield SM, Rosen HR, Tobler LH, Busch MP, McHutchison JG, Goldstein DB, Carrington M. 2009. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 461(7265):798-801.

Zhou Z, Hamming OJ, Ank N, Paludan SR, Nielsen AL, Hartmann R. 2007. Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases. J Virol 81(14):7749-58.



CHAPTER 3

IFN-Λ IS ABLE TO AUGMENT TLR-MEDIATED ACTIVATION AND SUBSEQUENT FUNCTION OF PRIMARY HUMAN B CELLS

Rik A. de Groen[1], Zwier M.A. Groothuismink[1], Bi-Sheng Liu[1,2], and André Boonstra[1]

[1] Department of Gastroenterology and Hepatology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[2] Department of Rheumatology, Leiden University Medical Center, Leiden, the Netherlands

J LEUKOC BIOL. 2015 OCT;98(4):623-30.

ABSTRACT

Over the last decade, increased emphasis has been placed on finding alternatives to interferon (IFN)-α-based therapies. One such alternative, IFN-λ, has shown therapeutic promise in a variety of diseases, but research of this family of cytokines has primary been focused on their antiviral activities. The goal of this study was to investigate the role of IFN-λ in the regulation and modulation of B cell function. We show that similar to IFN-α, IFN-λ1 is able to augment TLR-mediated B cell activation, partially attributed to an upregulation of TLR7 expression, and that both naïve and memory B cells express the limiting type III IFN receptor complex, IFN-λR1. Furthermore, this IFN-λ-enhanced B cell activation resulted in increased cytokine and immunoglobulin production during TLR7 challenge, most prominently after the addition of helper T cell signals. Ultimately, these elevated cytokine and immunoglobulin levels could be partially attributed to the increase in proliferation of TLR7-challenged B cells by both type I and type III IFNs. These findings demonstrate the ability of IFN-λ to boost humoral immunity, an important attribute to consider for further studies on immunity to pathogens, vaccine development, and ongoing advancement of therapeutic strategies aimed at replacing IFN-α-based treated with IFN-λ.

INTRODUCTION

The antiviral activities of type III interferons (IFN) to a wide variety of viruses have been extensively studied in recent years. Potent antiviral effects have been described in hepatitis C virus (HCV), human immunodeficiency virus (HIV), influenza, and a myriad of other infections [1–3]. The family of type III IFNs, also known as IFN-λ, consists of 4 members, IFN-λ1, IFN-λ2, IFN-λ3 and IFN-λ4, that all bind to the same receptor heterodimer, consequently activating the signal transducers and activators of transcription 1 (STAT1) and STAT2, and inducing expression of IFN-stimulated genes (ISGs). Type III IFNs resemble the type I IFNs, IFN-α and IFN-β, in many ways; exhibiting overlapping antiviral activities and nearly identical activation of signaling downstream of their respective receptors [4–6]. This is not necessarily expected as type I IFNs and type III IFNs signal via distinct receptor complexes. The heterodimeric IFN-α receptor is ubiquitously expressed across divergent cell populations, whereas expression of the IFN-λ receptor appears to be more restricted and expression of the complete receptor has primarily been described on cells of epithelial origin, specifically epithelial cells, hepatocytes, and fibroblasts [6–8].

The expression of the IFN-λ receptor on many immune cells and their responsiveness to type III IFN is still largely unclear. Expression of the IFN-λ receptor on plasmacytoid dendritic cells (DC) has already been established [9], and we, as well as others, have demonstrated that monocyte-derived macrophages, but not monocytes, express the limiting IFN-λ receptor dimer, IFN-λR1, and are consequently responsive to stimulation by IFN-λ [10–12]. Interestingly, we showed that the effect of IFN-λ was distinct from that of IFN-α, with opposing effects of these types of IFNs observed on TLR-induced IL-12p40 production. Moreover, although IFN-α had a potent, direct effect on natural killer (NK) cells, IFN-λ was able to indirectly affect NK cells via macrophage-derived IL-12, due to the absence of a complete IFN-λ receptor complex on NK cells.

Type I IFNs have been shown to be strong modulators of multiple immune cell populations, including B cells. IFN-α exposure of B cells strongly promotes humoral immunity via enhanced survival, reduced apoptosis, and enhancement of activation, proliferation, and immunoglobulin production [13–15]. These effects are best observed when B cells are additionally stimulated via the B cell receptor (BCR), CD40, or toll-like receptors (TLRs) [16, 17]. IFN-α-induced enhancement has been shown to be beneficial for generation of neutralizing antibodies, especially during viral infection [18]. In contrast, excessive production of antibodies against DNA complexes in patients with systemic lupus erythematosus (SLE) is also, at least in part, the result of an undesired IFN-α effect on B cells [19].

However, unlike IFN-α, little information is available on the effects of IFN-λ on B cells. Various studies have demonstrated that B cells express the IFN-λ receptor [20], but the responsiveness of B cells has not been studied in detail. Multiple

myeloma B cells have been shown to proliferate in response to IFN-λ1 [21], and other studies have suggested a type I and II IFN independent mechanism of TLR7 up-regulation in B cells [22]. Therefore, to gain further insight into the effect of IFN-λ on the B cell compartment, we investigated in detail the role of IFN-λ on the regulation and modulation of human B cell function in comparison to IFN-α.

MATERIALS AND METHODS

B cell isolation and culture

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (Sanquin Blood Supply, Rotterdam, The Netherlands) using density gradient centrifugation (Ficoll-Paque, GE Healthcare, Uppsala, Sweden). Monocytes and B cells were then purified from the PBMC fraction using magnetic labeled CD14 or CD19 micro-beads (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively according to the protocol provided by the manufacturer. The purity of monocytes and B cells isolated and used in this study always exceeded 95%.

B cells were cultured in RPMI1640 medium (Lonza, Verviers Sprl, Belgium) with the supplementation of 10% heat-inactivated fetal calf serum (FCS, Sigma-Aldrich, St. Louis, MO, USA), penicillin and streptomycin (Gibco, Carlsbad, CA, USA), L-glutamate (Lonza), and 1M HEPES Buffer (Lonza).

CFSE labeling and proliferation assay

Isolated $CD19^+$ B cells were labeled using 0.1 μM CFSE (Vybrant® CFDA SE Cell Tracer Kit, Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. Labeled B cell were then cultured for 5 days in RPMI1640 medium (10% FCS; Lonza) with 10 ng/ml IFN-α2b (Intron A, Merck, Whitehouse Station, NJ, USA) or 100 ng/ml IFN-λ1 (Bristol-Myers Squibb, Wallingford, CT, USA) alone or in combination with TLR2 agonist Pam3CSK4 (100 ng/ml; InvivoGen, San Diego, CA, USA), TLR7/8 agonist R848 (100 ng/ml; Enzo Life Sciences, Farmingdale, NY, USA), or TLR9 agonist CpG-B (5 μg/ml; ODN 2006, InvivoGen). All cultures were performed with or without mimicked T cell help provided by soluble CD40 ligand (sCD40L, 100 ng/ml; R&D Systems, Minneapolis, MN, USA) and $F(ab')^2$ fragments of anti-IgM (αIgM, 5 μg/ml; Jackson ImmunoResearch, Suffolk, UK). Proliferation was measured by flow cytometry (FACS Canto II, BD, San Diego, CA, USA) and analyzed using FlowJo (Tree Star Incorporated, Ashland, OR, USA).

Stimulation of B cells and surface marker analysis

$0.5x10^6$ PBMC from healthy donors were stimulated with 10 ng/ml IFN-α2b (Intron A, Merck) or 100 ng/ml IFN-λ1 (Bristol-Myers Squibb) alone or in combination with R848 (100 ng/ml; Enzo Life Sciences) for 3, 6, and 24 hours. Cellular activation and surface marker expression was measured on $CD3^-/CD19^+/CD27^{+/-}$ B cells (anti-CD3-PE (UCHT1, Biolegend, San Diego, CA, USA), anti-CD19-APC-eFluor780 (HIB19, eBioscience, San Diego, CA, USA), and anti-CD27-FITC (L128, BD Biosciences) using flow cytometric analysis with anti-CD25-APC (2A3, BD Biosciences), anti-CD40-PE-Cy5.5 (5C3, eBioscience), anti-CD69-PE-Cy7 (TP1.55.3, eBioscience), anti-CD80-FITC (B9.12.1, Beckman Coulter, Breda, CA, USA), anti-CD86-Pacific Blue (IT2.2, Biolegend), anti-HLA-ABC-FITC (W6/32, Biolegend), and anti-HLA-DR-PerCP-Cy5.5 (LN3, eBioscience) using flow cytometry (FACS Canto II, BD Biosciences) and analyzed using FlowJo (Tree Star Incorporated). Titration for concentrations of IFN-λ1 and IFN-α and the subsequent effect on CD69 expression are depicted in Supplementary Figure 2A.

For cytokine and immunoglobulin production in supernatant, $0.5x10^6$ isolated $CD19^+$ B cells were stimulated with TLR agonists and IFN-α2b or IFN-λ1 as described above for 3, 5, and 7 days. All cultures were performed with or without mimicked T cell help, sCD40L (100 ng/ml; R&D Systems) and $F(ab')^2$ fragments of anti-IgM (5 μg/ml; Jackson ImmunoResearch).

Immunoassay for detection of cytokines and immunoglobulins in supernatants

The concentrations of various cytokines were measured in the supernatants of stimulated $CD19^+$ B cells by the use of sandwich ELISA specific for IL-6 and IL-10 (Ready-Set-Go Kits, eBioscience) with a sensitivity for both assays of 2 pg/ml. Immunoglobulins were measured in the supernatants of stimulated B cells using sandwich ELISA kits specific for IgG and IgM (Bethyl Laboratories, Montgomery, TX, USA) with a sensitivity of 7.8 ng/ml and 15.6 ng/ml, respectively.

Gene expression quantification

Total RNA was isolated from HEK293 R19, hepatocytes isolated from healthy donors livers, as well as flow cytometric sorted total $CD20^+$ B cells, $CD14^+$ monocytes, $CD20^+/CD27^-$ naïve B cells, $CD20^+/CD27^+$ memory B cells, and IFN-stimulated isolated $CD19^+$ B cells using the RNeasy kit (Qiagen, Hilden, Germany) and cDNA was prepared using the Primescript cDNA synthesis kit (Takara, Shiga, Japan) from 500 ng RNA. All real-time PCR reactions were performed using a MyIQ5 detection system (Bio-Rad, Hercules, CA, USA) and relative mRNA expression levels were calculated using the housekeeping gene GAPDH. The following primer/probe sets (Applied Biosystems, Carlsbad, CA,

USA) were used for the housekeeping gene and genes of interest: GAPDH (Hs00266705_g1), IFN-λR1 (Hs00417120_m1), IFN-αR1 (Hs01066115_m1) IFN-αR2 (Hs01022060_m1), Mx1 (Hs00895608_m1), OAS1 (Hs00973637_m1), and TLR7 (Hs00152971_m1).

Statistical analysis

Data is expressed as the mean value ± standard error of the mean (SEM) unless indicated otherwise. Data was analyzed with Prism 5.0 software (GraphPad Software Incorporated, San Diego, CA, USA) using the Mann-Whitney U test to compare variables between independent groups and the Spearman rank correlation coefficient test for nonparametric correlations. In all analyses, a 2-tailed P value of less than 0.05 (confidence interval of 95%) was considered statistically significant.

RESULTS

Both naïve and memory B cells express IFN-λR1 mRNA, and IFN-λ1 stimulation in combination with TLR7/8 ligation is able to activate B cells

Type I IFN stimulation has been shown to affect specific B cell TLR expression and responsiveness after challenge with their respective agonists [16, 23]. Since little is known about the immune effects of type III IFN, we chose to explore to what extent type III IFN are able to regulate B cell activation, alone or in combination with TLR stimulation, as well as how these compare to type I IFN. To do so, we stimulated human B cells for 3, 6, and 24 hours with either IFN-α, IFN-λ1, or IFN-α/IFN-λ1 alone or in combination with the TLR7/8 ligand R848 and performed flowcytometric analysis for an array of activation markers. Of the activation markers tested, only CD69 was found to be significantly upregulated by IFN-λ1 as well as IFN-α, but only in combination with R848 triggering (Figure 1A-B). This was true for both the frequency of CD69-expressing B cells as well as for the mean fluorescent intensity (MFI) of CD69 expression levels on B cells (Supplementary Figure 1A-B). IFN-α, however, was able to already induce significantly increased CD69 expression at 3 hours, while significant effects of IFN-λ1 were only observed after 6 hours. IFN-α alone was also able to significantly induce CD69 expression, something not observed for IFN-λ1 stimulation. However, CD25 expression (Figure 1A), CD40, CD80, CD86, HLA-ABC, and HLA-DR (data not shown) were all unaffected by IFN-λ1 stimulation, with or without the addition of TLR challenge. Similar results were observed when analyzing $CD27^-$ naïve and $CD27^+$ B cells independently (Supplementary Figure 2B), suggesting that both naïve and memory B cell populations are responsive to IFN-λ1 stimulation.

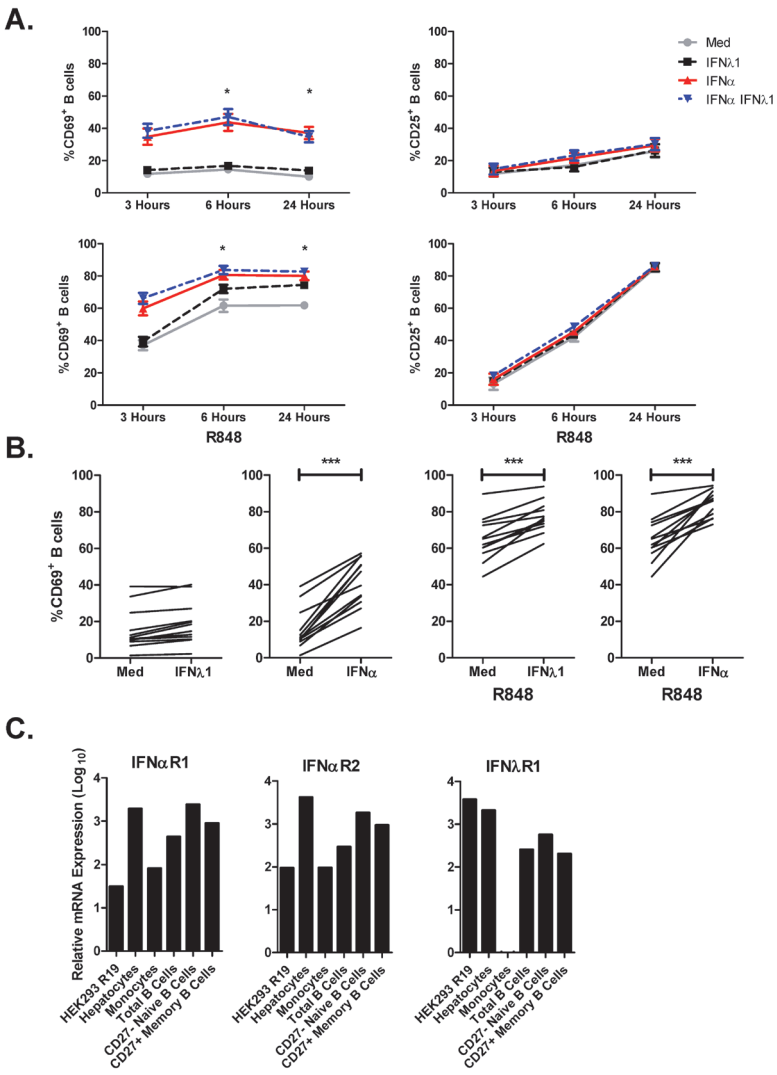


Figure 1. Both naïve and memory B cells express IFN-λR1 mRNA, and IFN-λ1 stimulation in combination with TLR7/8 ligation is able to activate B cells

(A) PBMCs (n = 6) were stimulated 3, 6, or 24 hours with IFN-λ or IFN-α, with or without the combination of TLR7/8 agonist R848, and flow cytometric analysis was performed to determine the expression of the activation markers CD69 and CD25 on $CD3^-/CD19^+$ B cells. (B) Individual (n = 12) results for CD69 expression on B cells after 24 hours IFN/R848 stimulation. (C) Representative gene expression of IFN-λR1, IFN-αR1, and IFN-αR2 mRNA by sorted primary resting monocytes, total B cells, $CD27^-$ naïve B cells, and $CD27^+$ memory B cells. The relative mRNA expression was measured by qPCR, using primary hepatocytes and HEK293 cells stably transfected to express IFN-λR1 (HEK293R19) as positive controls. Expression levels are shown as the ΔΔCT relative to the housekeeping gene GAPDH. Data are shown as mean ± SEM and are representative of 4 (A-B) independent experiments.*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ as compared to the control condition (Mann-Whitney U test).

To determine if type I and type III IFNs are able to act on equivalent B cell populations, naïve and memory B cells were sorted based on their CD27 expression, and qPCR analysis was performed for the expression of their respective receptors. A HEK293 cell line (HEK293 R19) stably transfected to express IFN-λR1 mRNA as well as primary human hepatocytes, known to express the receptor and respond to IFN-λ [6], were included as comparative positive controls, while the primary human monocytes were included as negative controls. Both IFN-λR1 and IFN-αR1/2 mRNA expression were found in naïve $CD27^-$ and memory $CD27^+$ B cells, using total sorted B cells as a comparative control and sorted $CD14^+$ monocytes as negative control for IFN-λR1 expression (Figure 1C). Collectively this data shows that similar to type I IFN, type III IFN are able to induce B cell activation, in combination TLR7/8 challenge, and that both naïve and memory B cells could be perspective targets.

IFN-λ1 stimulation induces ISG and TLR7 expression in primary human B cells

The ability of IFN-α to enhance TLR7 sensitivity of B cells by selectively upregulating TLR7 expression has been previously described [16]. To determine if IFN-λ1 resembles type I IFNs in this ability, and to confirm the ability of B cells to respond to type III IFNs, isolated B cells were stimulated with either IFN-α, IFN-λ, or a combination of IFN-α/IFN-λ1, and qPCR was performed for the expression of ISGs Mx1 and OAS1, TLR7, and IFN-λR1. Both IFN-λ1 and IFN-α were able to significantly upregulate MX1 and OAS1 mRNA expression (Figure 2A), confirming B cell responsiveness to both type I and III IFNs. IFN-λ1 and IFN-α were also both able to significantly upregulate TLR7 expression (Figure 2B), a potential explanation for the enhanced activation observed in response to TLR7/8 stimulation during co-culture with IFN-λ1. IFN-λR1 expression, however, was only slightly, but significantly, upregulated by IFN-α stimulation, similar to the regulation by IFN-α on IFN-λR1 expression described in hepatocytes [24]. The combined stimulation of IFN-α and IFN-λ1 had similar effects as compared to the individual cytokines with only a minimal synergistic effect.

IFN-λ1 enhances TLR7/8-induced immunoglobulin production and TLR9-induced cytokine production of B cells

With the confirmed expression of the IFN-λ receptor on both naïve and memory B cells and observed increased activation after TLR7/8 stimulation, the next step was to determine to what extent IFN-λ1 was able to modulate B cell function. This was done by stimulating isolated B cells with either IFN-α or IFN-λ1 in combination with R848, and assessing immunoglobulin and cytokine production at days 3, 5, or 7 using immunosorbent assays. As expected, both IFN-λ1 and IFN-α were able to enhance TLR7/8-mediated IgG and IgM production in isolated B cells,

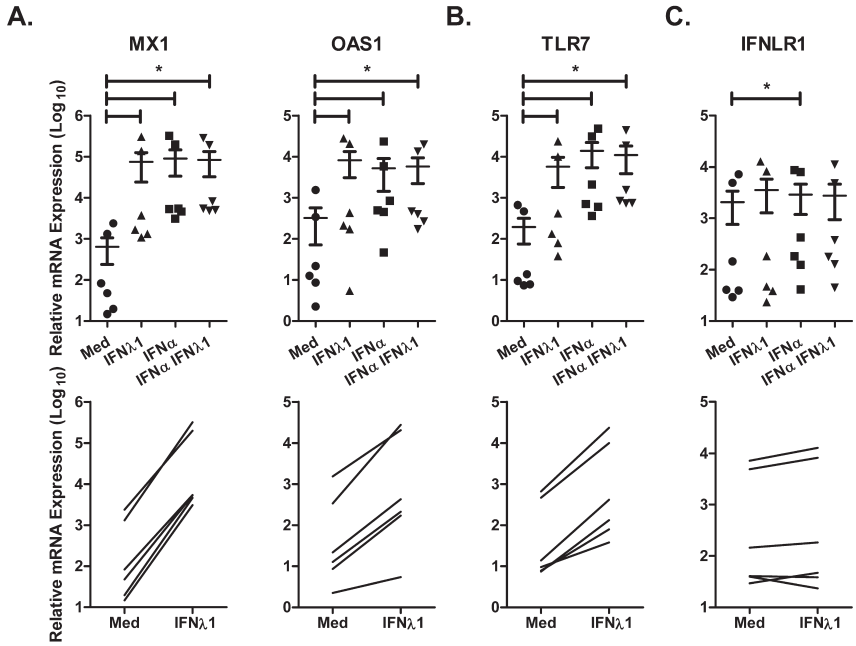


Figure 2. IFN-λ1 stimulation induces ISG and TLR7 expression in primary human B cells
(A) Purified B cells (n = 6) were stimulated with either IFN-α, IFN-λ, or a combination of IFN-α/IFN-λ1 for 3 hours, and qPCR was performed for the expression of ISGs MX1 and OAS1, (B) TLR7, and (C) IFN-λR1. Expression levels are shown as the ΔΔCT relative to the housekeeping gene GAPDH. Data are shown as mean ± SEM and are representative of 2 independent experiments.*$P < 0.05$ and **$P < 0.01$, as compared to the control condition (Mann-Whitney U test).

and increased consistently over the culture period, with highest augmentation observed at day 7 (Figure 3A). The effect of IFN-λ1 or IFN-α on stimulation in the presence of TLR2 (Pam3CSK4) and TLR9 (CpG) differed from the cultures containing R848; Pam3CSK4 was unable to induce IgG or IgM production, and CpG-mediated immunoglobulin production was unaltered by the addition of either type I or III IFN (Figure 3A).

Concurrently to the immunoglobulin analysis, isolated B cells were also evaluated for their cytokine production upon IFN and TLR stimulation. After culturing for 3 days, IFN-λ1 showed a strong trend toward enhancement of R848-mediated IL-6 ($p = 0.055$), whereas no effect on IL-10 levels was observed (Figure 3B). In contrast, both R848 and CpG-mediated IL-6 production were significantly increased by IFN-α at day 3. At day 7, IL-6 and IL-10 levels were significantly altered by IFN-α in R848 co-stimulations, and only IL-6 was enhanced by both IFN-α and IFN-λ in combination with CpG. These results implicate a role for both IFN-α and IFN-λ in the modulation of TLR-triggered cytokine and immunoglobulin production of B cells.

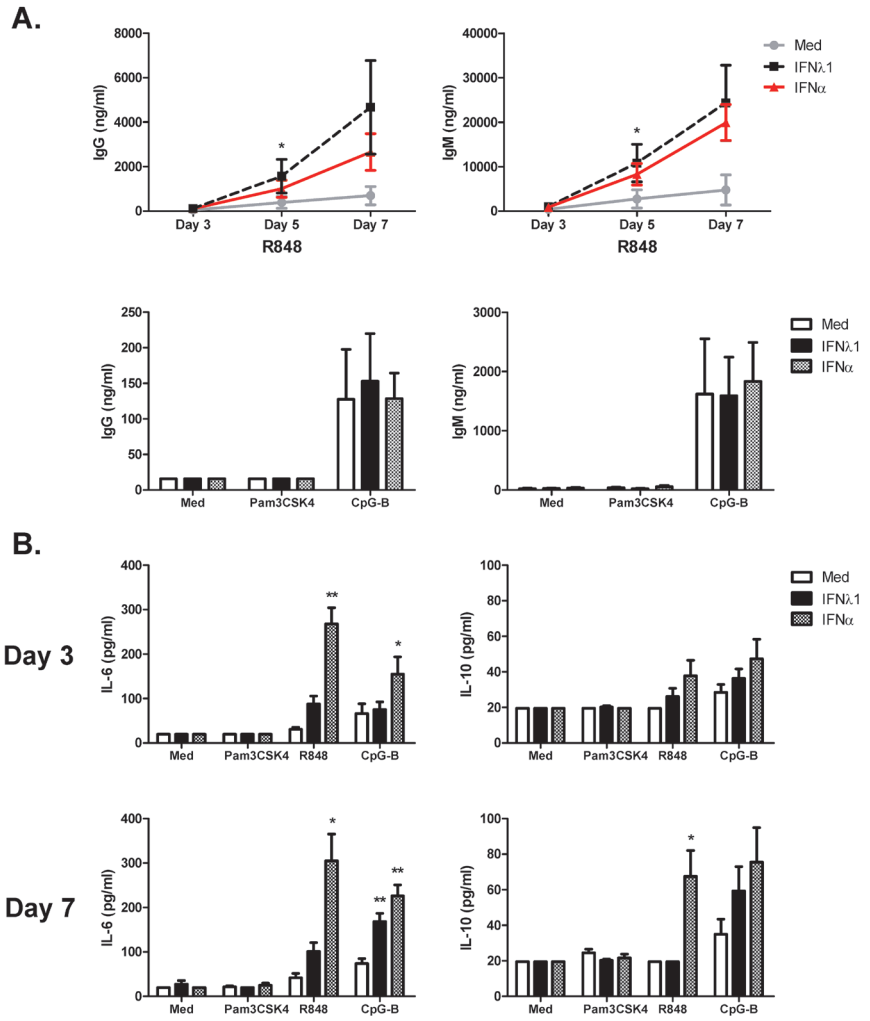


Figure 3. IFN-λ1 enhances TLR7/8-induced immunoglobulin production and TLR9-induced cytokine production by B cells

(A) Purified B cells (n = 5) were stimulated with R848 in combination with either IFN-α or IFN-λ, and supernatants were harvested at days 3, 5, and 7 for ELISA analysis of IgG and IgM. Identical cultures were performed using medium, Pam3CSK, and CpG-B and results are shown for day 7 of stimulation. (B) IL-6 and IL-10 levels were determined by ELISA of the aforementioned B cell cultures using medium, Pam3CSK, R848 or CpG-B stimulation supplemented with either IFN-α or IFN-λ after 3 and 7 days. Data are shown as mean ± SEM and are representative of 3 independent experiments.*$P < 0.05$ and **$P < 0.01$ as compared to the control condition (Mann-Whitney U test).

BCR and CD40 ligation are able to further increase IFN/TLR-mediated cytokine production of human B cells

After observing that type III IFN was able to promote TLR-mediated B cell antibody and cytokine production, we were interested to study if this modulation could be further affected by additional B cell signals. TLR and antigen receptors have been well-established integrators of B cell responses, including but not limited to the production of IL-6 and IL-10 as well as a broad range of immunoglobulin subclasses.[25] We therefore collected supernatants after 3 and 7 days of TLR stimulated B cells cultured in the presence of IFN-α or IFN-λ1, this time with simulated T cell help in the form of CD40 ligation and triggering of the B cell receptor via IgM. The addition of sCD40L and anti-IgM (αIgM) significantly enhanced the levels of IL-6 and IL-10 in supernatants of B cells stimulated with IFN-λ1 at day 3 and 7 alongside of TLR7/8 and TLR9 challenge (except for IL-10 day 7 IFN-λ1/R848, $p = 0.055$) (Figure 4). IFN-α had a similar effect with significant or strong trends for increased TLR7/8 and TLR9-mediated IL-6 and IL-10 production by B cells exposed to mimicked T cell help at both day 3 and 7. The importance of additional T cell help for robust cytokine production can be explained by the predominance of naïve B cells in the peripheral blood compartment, and the previously established requirement of additional signals for naïve B cell activation.[17]

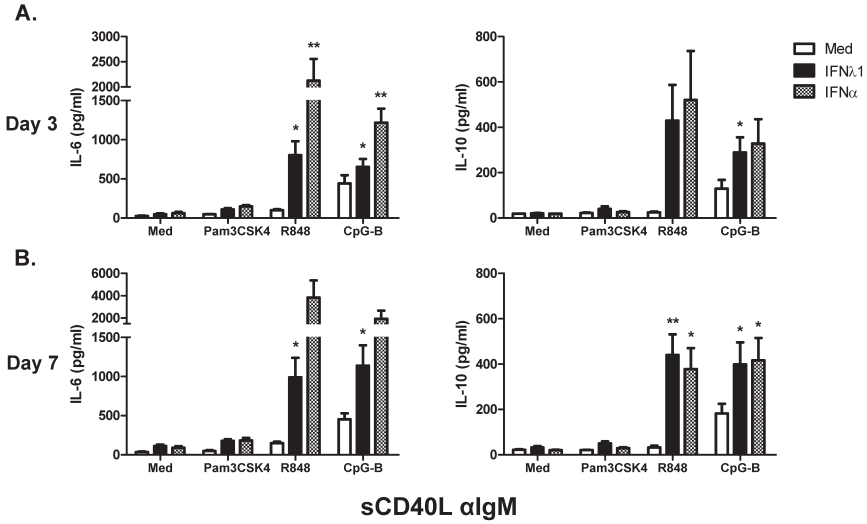


Figure 4. BCR and CD40 ligation further increase IFN/TLR-mediated cytokine production by human B cells

(A, B) Purified B cells ($n = 5$) were stimulated with an array of TLR agonists, Pam3CSK, R848 or CpG-B, in combination with mimicked T cell help, using sCD40L and αIgM. The cultures were supplemented with either IFN-α or IFN-λ and supernatants were harvested at day 3 and day 7 for ELISA analysis of IL-6 and IL-10 production. Data are shown as mean ± SEM and are representative of 3 independent experiments.*$P < 0.05$ and **$P < 0.01$ as compared to the control condition (Mann-Whitney U test).

Similar to type I IFN, type III IFN enhance TLR and CD40/BCR-mediated B cell proliferation

The increased cytokine and immunoglobulin production observed by type I and III IFN stimulation led us to examine the possibility that these IFN-induced effects may be the result of enhanced B cell proliferation. To do so, we stimulated CFSE-labeled B cells with Pam3CSK4, R848, or CpG alone or in combination with IFN-α or IFN-λ for 5 days (Figure 5A). Only TLR9 (CpG) challenge alone was able to induce B cell proliferation without the addition of either IFN. The addition of type I as well as type III IFNs enhanced R848-induced B cell proliferation, but did not further increase CpG-induced responses. As expected, the additional B cell triggering using CD40-ligand and BCR ligation augmented the proliferative responses. The frequency of proliferated B cells upon CD40 and BCR stimulation (Figure 5A, B) was increased upon additional R848 and CpG stimulation.

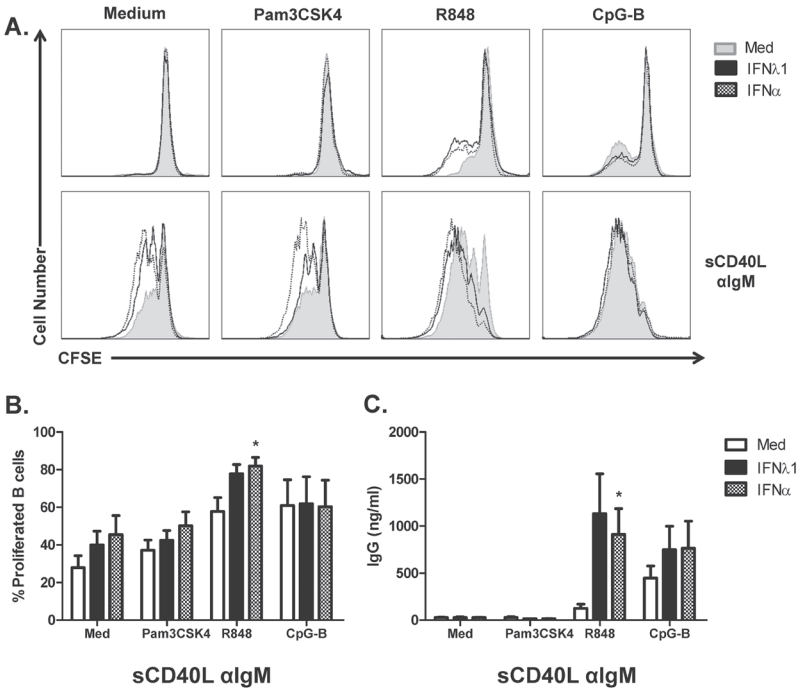


Figure 5. Similar to type I IFN, type III IFN enhances TLR and BCR-mediated B cell proliferation (A) CFSE-labeled human B cells were cultured in the presence of Pam3CSK4, R848, or CpG-B in combination with IFN-α or IFN-λ1 for a total of 5 days. sCD40L and αIgM were added to a fraction of the cultures, and the results were analyzed using flow cytometry. Shown are histograms of a representative for B cell proliferation (B) and collective results of multiple donors (n = 4). (C) Accompanying IgG production measured in the supernatant of B cells after 7 days of stimulation (n = 5). Data are shown as mean ± SEM and are representative of 3 independent experiments.*$P < 0.05$ and **$P < 0.01$ as compared to the control condition (Mann-Whitney U test).

However, R848-induced, but not CpG-induced, proliferative responses were significantly increased by IFN-α as well as IFN-λ1. The higher levels of B cell proliferation upon stimulation of R848 and IFN-α or IFN-λ1 in combination with CD40/BCR ligation were mirrored by a significant increase of IgG production in supernatants of these cells (Figure 5C). Collectively, these results confirm a role of both IFN-α and IFN-λ1 in the enhancement of TLR7/8-mediated B cell proliferation and IgG production. TLR9 stimulation, although being the most effective inducer of proliferation alone, was independent and unaffected by the addition of either type of IFNs.

DISCUSSION

The focus of this study was to examine the ability of IFN-λ to modulate the activity and function of human B cells, both alone and in combination with specific TLR activation. We demonstrate that similar to IFN-α, IFN-λ1 is able to augment TLR-mediated B cell activation, but only in combination with TLR7/8 or TLR9 ligation. In contrast to IFN-α, IFN-λ1 stimulation alone was unable to activate B cells, indicated by the lack of induction of CD69 expression. Both naïve and memory B cells expressed the limiting type III IFN receptor component, IFN-λR1, as well the complete IFN-αR1/2 complex. IFN-λ1 stimulation alone was however sufficient to induce ISGs Mx1 and OAS1 as well as TLR7 expression in B cells. We further show that this IFN-λ-enhanced B cell activation resulted in increased cytokine and immunoglobulin production during both TLR7/8 and TLR9 challenge, most prominently after the addition of helper T cell signals. Ultimately, these elevated cytokine and immunoglobulin levels could be partially attributed to the increase in proliferation of TLR7/8-challenged B cells by both type I and type III IFNs.

Previous studies have investigated and established the effects of type I IFNs on B cells [13–15] and its ability to enhance TLR7 sensitivity of B cells by selectively up-regulating TLR7 expression [16]. However, the effects of type III IFNs on B cell TLR response and function still remain relatively unexplored, with descriptions of non-type I and II IFN enhancement of TLR7 prospectively attributed to IFN-λ [22]. Our study is the first to definitely demonstrate that this ability to enhance TLR-induced responses in B cells, partially attributed to an upregulation of TLR7 expression, is in fact also a characteristic of IFN-λ. The upregulation of ISGs and TLR7 observed after the combined stimulation of IFN-α and IFN-λ1 resembled that of either IFN independently, most likely due to the overlapping signaling cascades described for type I and III IFNs [26, 27]. However, although stimulation with either IFN alone was able to induce Mx1 and OAS1 expression, one aspect where IFN-λ differed from IFN-α, was in its inability to independently (without the addition of TLR challenge) activate B cells, assessed by CD69 expression. This immediate action of IFN-α has been shown to be integral in the early activation of

B cells during influenza infection, mediating activation, transcriptional response, and the antibody responses [18]. IFN-λ, however, has previously been described to be kinetically distinct and has a more delayed effect on specific cellular populations than that of IFN-α [28, 29]. These divergences in kinetics and its requirement of additional signals to activate B cells suggest that IFN-λ could represent a more postponed and specific means of B cell activation compared to that of IFN-α.

Type I IFNs have been shown to act on all populations of B cells, but this is not the case for TLR expression and response in B cells. Higher levels of TLR7 and TLR9 expression and increased responses of their respective ligands have been reported on memory B cells as compared to naïve B cells [30, 31]. We demonstrate that, similar to IFN-αR1/2, IFN-λR1 is expressed at comparable levels on both $CD27^-$ naïve B cell and $CD27^+$ memory B cell subsets and upregulate CD69 expression upon TLR7/8 challenge in combination with IFN-λ1 stimulation, indicating that type I and III IFNs are similar in their targeting of B cells at different activation stages. This suggests that the necessity of an additional TLR signal for the induction of B cell activation by IFN-λ compared to IFN-α is not the result of targeting differing subsets, and that expression of the IFN-λ receptor is not associated with B cell memory phenotype, as described for TLR expression.

DCs have been described as potent producers of both IFN-α and IFN-λ, with IFN-α production primarily attributed to plasmacytoid DCs and IFN-λ production to $BDCA3^+$ myeloid DCs [32–36]. As previously discussed, DC-derived IFN-α has been shown to increase B cell responsiveness to TLR7 stimulation, and more recent studies have suggested similar roles for IFN-λ in the up-regulation of B cell TLR7 expression as well as in their capacity to produce immunoglobulins [22, 37]. In our study, we now conclusively establish that IFN-λ, in combination with TLR7 challenge, was able indeed to induce B cell activation and subsequently increase immunoglobulin production, as well as cytokine production, which were partially attributed to enhanced B cell proliferation. Interestingly, we also observed an increase in IL-6 and IL-10 production by B cells stimulated with IFN-λ in combination with CpG, but in contrast to R848 stimulation, had no effect on proliferation or immunoglobulin production. This implies an additional mechanism, apart from the effect of IFN-λ on B cell proliferation, for the enhancement of TLR-induced cytokine production by B cells. Although initially observed in combination with TLR9 challenge, this does not have to be limited to TLR9 stimulation, as this effect may act in conjunction with the effects of B cell proliferation. IL-6 and IL-10 production have been shown to be a drivers of B cell proliferation and/or immunoglobulin production, and key factors in the observed hyperactivity of B cells in SLE patients, leading to immune complexes and tissue and organ damage [38–40]. The promotion of the immune response by IFN-λ resembles our previous observations in macrophages, where IFN-λ was able to promote IL-12p40 and TNF production upon TLR4 and TLR7/8 stimulation [10, 11]. Although, IFN-λ1 was specifically used in this study with the goal of understanding this specific

cytokine's therapeutic potential, our previous studies have shown the IFN-λ2–3 had nearly identical effects to IFN-λ1 on monocyte-derived macrophages, due to their comparable receptor affinities.

The continued investigation of IFN-λ and its immunomodulatory capacities, independent of direct antiviral activities, is integral in understanding this class of cytokines and the role it plays in antiviral immunity. In this study, we were able to demonstrate the effects of IFN-λ on B cells, specifically in combination with TLR ligation, an important attribute for future studies to consider when determining the role of IFN-λ in the controlling of infections, but also for the exploration of the therapeutic potential of IFN-λ in treatment of chronic hepatitis B infection, as well in ongoing efforts to advance vaccine efficacies.

AUTHORSHIP

R.A. de Groen designed research, performed research, analyzed data, and wrote the article; Z.M.A. Groothuismink performed research and analyzed data; B-S. Liu designed research and wrote the article; A. Boonstra designed research, analyzed data, and wrote the article.

ACKNOWLEDGMENTS

This study was supported by the Virgo consortium, funded by the Dutch government project number FES0908, and by the Netherlands Genomics Initiative (NGI) project number 050–060–452; and further supported by funding of Bristol-Myers Squibb.

DISCLOSURE OF CONFLICTS OF INTEREST

This study was partly financially supported by Bristol-Myers Squibb (BMS). This, however, has not influenced the conclusions or the data presented in the current article.

ETHICAL APPROVAL

This study was conducted according to the principles expressed in the Declaration of Helsinki. All human material was collected from healthy donors, and written informed consent was obtained from all individuals prior to their inclusion.

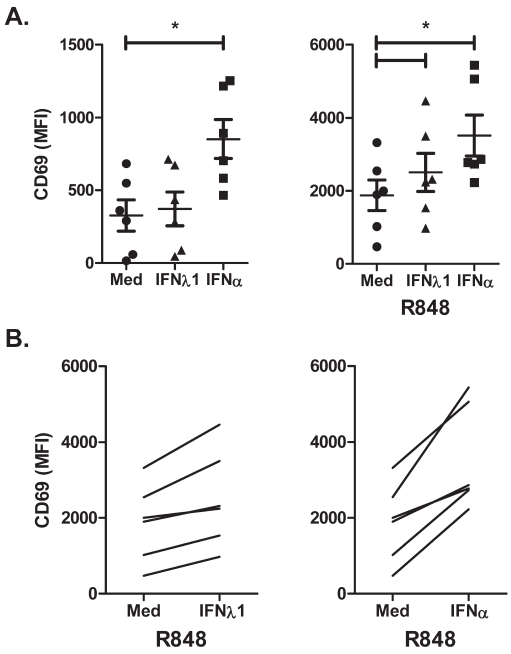
REFERENCES

1. Tian, R. R., Guo, H. X., Wei, J. F., Yang, C. K., He, S. H., Wang, J. H. (2012) IFN-lambda inhibits HIV-1 integration and post-transcriptional events in vitro, but there is only limited in vivo repression of viral production. Antiviral Res 95, 57-65.
2. Egli, A., Santer, D. M., O'Shea, D., Barakat, K., Syedbasha, M., Vollmer, M., Baluch, A., Bhat, R., Groenendyk, J., Joyce, M. A., Lisboa, L. F., Thomas, B. S., Battegay, M., Khanna, N., Mueller, T., Tyrrell, D. L., Houghton, M., Humar, A., Kumar, D. (2014) IL-28B is a Key Regulator of B- and T-Cell Vaccine Responses against Influenza. PLoS Pathog 10, e1004556.
3. Pagliaccetti, N. E., Eduardo, R., Kleinstein, S. H., Mu, X. J., Bandi, P., Robek, M. D. (2008) Interleukin-29 functions cooperatively with interferon to induce antiviral gene expression and inhibit hepatitis C virus replication. J Biol Chem 283, 30079-89.
4. Zhou, Z., Hamming, O. J., Ank, N., Paludan, S. R., Nielsen, A. L., Hartmann, R. (2007) Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases. J Virol 81, 7749-58.
5. Dumoutier, L., Tounsi, A., Michiels, T., Sommereyns, C., Kotenko, S. V., Renauld, J. C. (2004) Role of the interleukin (IL)-28 receptor tyrosine residues for antiviral and antiproliferative activity of IL-29/interferon-lambda 1: similarities with type I interferon signaling. J Biol Chem 279, 32269-74.
6. Doyle, S. E., Schreckhise, H., Khuu-Duong, K., Henderson, K., Rosler, R., Storey, H., Yao, L., Liu, H., Barahmand-pour, F., Sivakumar, P., Chan, C., Birks, C., Foster, D., Clegg, C. H., Wietzke-Braun, P., Mihm, S., Klucher, K. M. (2006) Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. Hepatology 44, 896-906.
7. Ank, N., Iversen, M. B., Bartholdy, C., Staeheli, P., Hartmann, R., Jensen, U. B., Dagnaes-Hansen, F., Thomsen, A. R., Chen, Z., Haugen, H., Klucher, K., Paludan, S. R. (2008) An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. J Immunol 180, 2474-85.
8. Xu, L., Feng, X., Tan, W., Gu, W., Guo, D., Zhang, M., Wang, F. (2013) IL-29 enhances Toll-like receptor-mediated IL-6 and IL-8 production by the synovial fibroblasts from rheumatoid arthritis patients. Arthritis Res Ther 15, R170.
9. Megjugorac, N. J., Gallagher, G. E., Gallagher, G. (2009) Modulation of human plasmacytoid DC function by IFN-lambda1 (IL-29). J Leukoc Biol 86, 1359-63.
10. de Groen, R. A., Boltjes, A., Hou, J., Liu, B. S., McPhee, F., Friborg, J., Janssen, H. L., Boonstra, A. (2015) IFN-lambda-mediated IL-12 production in macrophages induces IFN-gamma production in human NK cells. Eur J Immunol 45, 250-9.
11. Liu, B. S., Janssen, H. L., Boonstra, A. (2011) IL-29 and IFNalpha differ in their ability to modulate IL-12 production by TLR-activated human macrophages and exhibit differential regulation of the IFNgamma receptor expression. Blood 117, 2385-95.
12. Liu, M. Q., Zhou, D. J., Wang, X., Zhou, W., Ye, L., Li, J. L., Wang, Y. Z., Ho, W. Z. (2012) IFN-lambda3 inhibits HIV infection of macrophages through the JAK-STAT pathway. PLoS One 7, e35902.

13. Le Bon, A., Schiavoni, G., D'Agostino, G., Gresser, I., Belardelli, F., Tough, D. F. (2001) Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. Immunity 14, 461-70.
14. Le Bon, A., Thompson, C., Kamphuis, E., Durand, V., Rossmann, C., Kalinke, U., Tough, D. F. (2006) Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. J Immunol 176, 2074-8.
15. Swanson, C. L., Wilson, T. J., Strauch, P., Colonna, M., Pelanda, R., Torres, R. M. (2010) Type I IFN enhances follicular B cell contribution to the T cell-independent antibody response. J Exp Med 207, 1485-500.
16. Bekeredjian-Ding, I. B., Wagner, M., Hornung, V., Giese, T., Schnurr, M., Endres, S., Hartmann, G. (2005) Plasmacytoid dendritic cells control TLR7 sensitivity of naive B cells via type I IFN. J Immunol 174, 4043-50.
17. Ruprecht, C. R. and Lanzavecchia, A. (2006) Toll-like receptor stimulation as a third signal required for activation of human naive B cells. European Journal of Immunology 36, 810-816.
18. Coro, E. S., Chang, W. L., Baumgarth, N. (2006) Type I IFN receptor signals directly stimulate local B cells early following influenza virus infection. J Immunol 176, 4343-51.
19. Niewold, T. B., Clark, D. N., Salloum, R., Poole, B. D. (2010) Interferon alpha in systemic lupus erythematosus. J Biomed Biotechnol 2010, 948364.
20. Witte, K., Gruetz, G., Volk, H. D., Looman, A. C., Asadullah, K., Sterry, W., Sabat, R., Wolk, K. (2009) Despite IFN-lambda receptor expression, blood immune cells, but not keratinocytes or melanocytes, have an impaired response to type III interferons: implications for therapeutic applications of these cytokines. Genes Immun 10, 702-14.
21. Novak, A. J., Grote, D. M., Ziesmer, S. C., Rajkumar, V., Doyle, S. E., Ansell, S. M. (2008) A role for IFN-lambda1 in multiple myeloma B cell growth. Leukemia 22, 2240-6.
22. Sinha, S., Guo, Y., Thet, S., Yuan, D. (2012) IFN type I and type II independent enhancement of B cell TLR7 expression by natural killer cells. J Leukoc Biol 92, 713-22.
23. Bourke, E., Bosisio, D., Golay, J., Polentarutti, N., Mantovani, A. (2003) The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. Blood 102, 956-63.
24. Duong, F. H., Trincucci, G., Boldanova, T., Calabrese, D., Campana, B., Krol, I., Durand, S. C., Heydmann, L., Zeisel, M. B., Baumert, T. F., Heim, M. H. (2014) IFN-lambda receptor 1 expression is induced in chronic hepatitis C and correlates with the IFN-lambda3 genotype and with nonresponsiveness to IFN-alpha therapies. J Exp Med 211, 857-68.
25. Rawlings, D. J., Schwartz, M. A., Jackson, S. W., Meyer-Bahlburg, A. (2012) Integration of B cell responses through Toll-like receptors and antigen receptors. Nature Reviews Immunology 12, 282-294.
26. Donnelly, R. P. and Kotenko, S. V. (2010) Interferon-lambda: a new addition to an old family. J Interferon Cytokine Res 30, 555-64.
27. Rauch, I., Muller, M., Decker, T. (2013) The regulation of inflammation by interferons and their STATs. JAKSTAT 2, e23820.
28. Jilg, N., Lin, W., Hong, J., Schaefer, E. A., Wolski, D., Meixong, J., Goto, K., Brisac, C., Chusri, P., Fusco, D. N., Chevaliez, S., Luther, J., Kumthip, K., Urban, T. J., Peng, L. F., Lauer, G. M., Chung, R. T. (2014) Kinetic differences in the induction of interferon

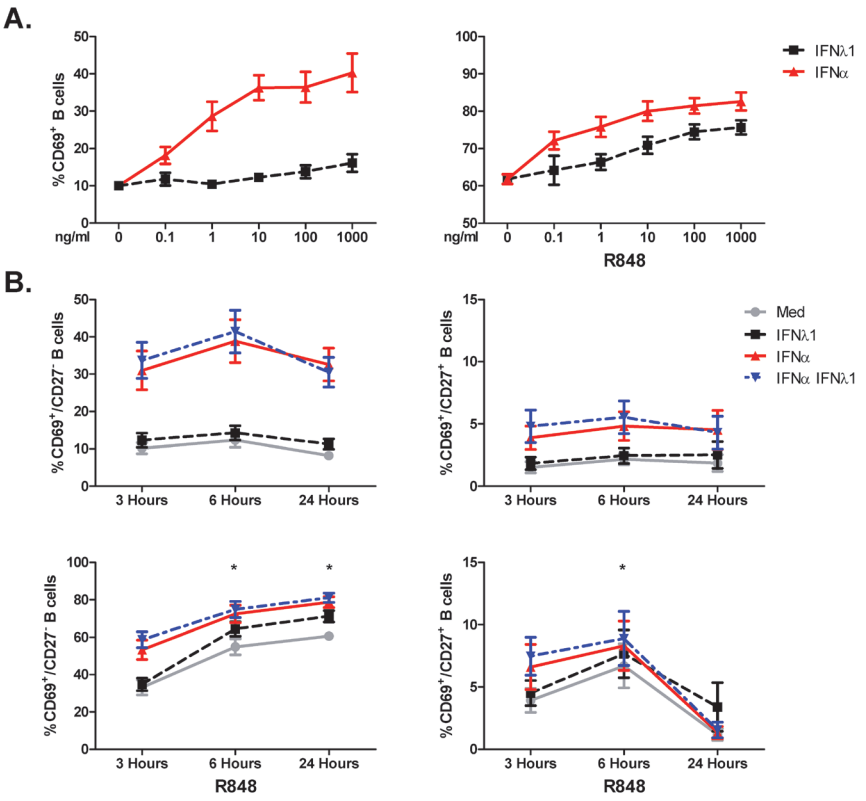
stimulated genes by interferon-alpha and interleukin 28B are altered by infection with hepatitis C virus. Hepatology 59, 1250-61.

29. Olagnier, D. and Hiscott, J. (2014) Type I and type III interferon-induced immune response: it's a matter of kinetics and magnitude. Hepatology 59, 1225-8.
30. Bernasconi, N. L., Onai, N., Lanzavecchia, A. (2003) A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood 101, 4500-4.
31. Agrawal, S. and Gupta, S. (2011) TLR1/2, TLR7, and TLR9 signals directly activate human peripheral blood naive and memory B cell subsets to produce cytokines, chemokines, and hematopoietic growth factors. J Clin Immunol 31, 89-98.
32. Siegal, F. P., Kadowaki, N., Shodell, M., Fitzgerald-Bocarsly, P. A., Shah, K., Ho, S., Antonenko, S., Liu, Y. J. (1999) The nature of the principal type 1 interferon-producing cells in human blood. Science 284, 1835-7.
33. Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A., Colonna, M. (1999) Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nat Med 5, 919-23.
34. Lauterbach, H., Bathke, B., Gilles, S., Traidl-Hoffmann, C., Luber, C. A., Fejer, G., Freudenberg, M. A., Davey, G. M., Vremec, D., Kallies, A., Wu, L., Shortman, K., Chaplin, P., Suter, M., O'Keeffe, M., Hochrein, H. (2010) Mouse CD8alpha+ DCs and human BDCA3+ DCs are major producers of IFN-lambda in response to poly IC. J Exp Med 207, 2703-17.
35. Yoshio, S., Kanto, T., Kuroda, S., Matsubara, T., Higashitani, K., Kakita, N., Ishida, H., Hiramatsu, N., Nagano, H., Sugiyama, M., Murata, K., Fukuhara, T., Matsuura, Y., Hayashi, N., Mizokami, M., Takehara, T. (2013) Human blood dendritic cell antigen 3 (BDCA3)(+) dendritic cells are a potent producer of interferon-lambda in response to hepatitis C virus. Hepatology 57, 1705-15.
36. van der Aa, E., van de Laar, L., Janssen, H. L., van Montfoort, N., Woltman, A. M. (2015) BDCA3 expression is associated with high IFN-lambda production by CD34 -derived dendritic cells generated in the presence of GM-CSF, IL-4 and/or TGF-beta. Eur J Immunol.
37. Hummelshoj, L., Ryder, L. P., Nielsen, L. K., Nielsen, C. H., Poulsen, L. K. (2006) Class switch recombination in selective IgA-deficient subjects. Clin Exp Immunol 144, 458-66.
38. Malisan, F., Briere, F., Bridon, J. M., Harindranath, N., Mills, F. C., Max, E. E., Banchereau, J., Martinez-Valdez, H. (1996) Interleukin-10 induces immunoglobulin G isotype switch recombination in human CD40-activated naive B lymphocytes. J Exp Med 183, 937-47.
39. Friman, V., Hanson, L. A., Bridon, J. M., Tarkowski, A., Banchereau, J., Briere, F. (1996) IL-10-driven immunoglobulin production by B lymphocytes from IgA-deficient individuals correlates to infection proneness. Clin Exp Immunol 104, 432-8.
40. Moore, K. W., de Waal Malefyt, R., Coffman, R. L., O'Garra, A. (2001) Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 19, 683-765.



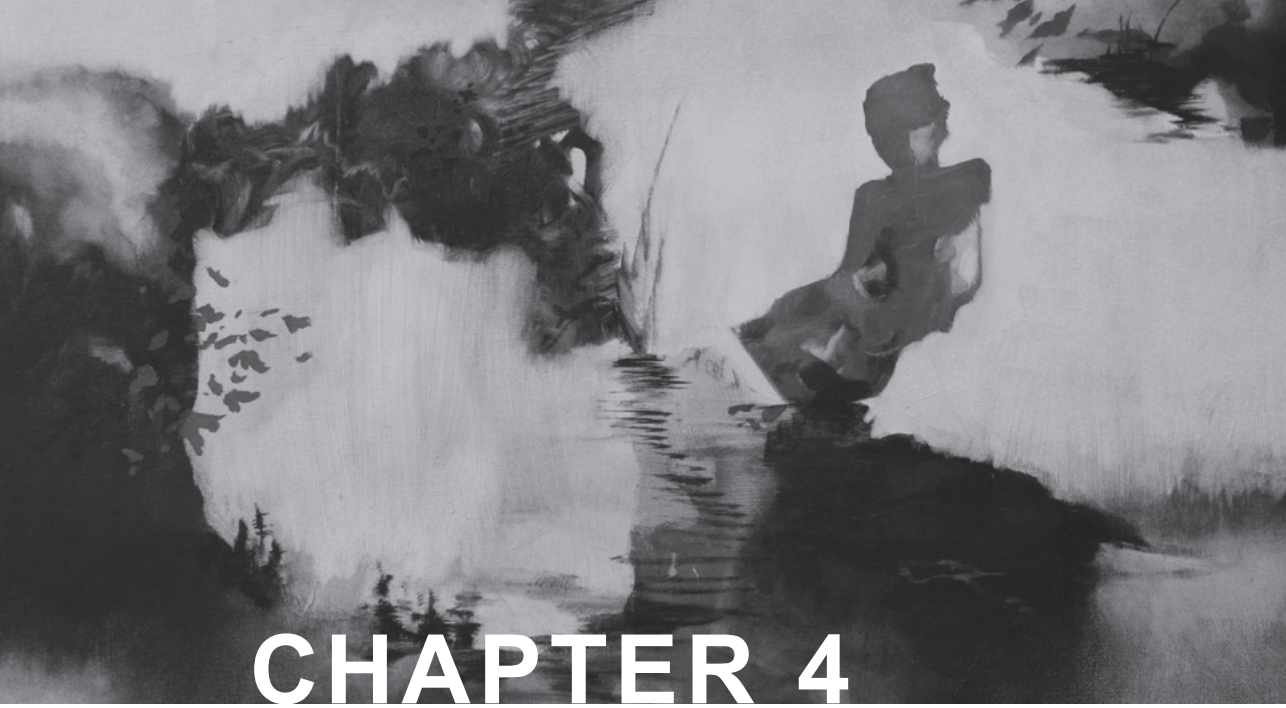
Supplementary Figure 1. IFN-λ1 stimulation in combination with TLR7/8 ligation is able to activate B cells

(A) PBMCs (n = 6) were stimulated 3, 6, or 24 hours with IFN-λ or IFN-α, with or without the combination of TLR7/8 agonist R848, and flow cytometric analysis was performed to determine the expression of the activation markers CD69 on $CD3^-/CD19^+$ B cells. (B) Individual (n = 6) results for CD69 MFI on B cells after 24 hours IFN/R848 stimulation. Data are shown as mean ± SEM and are representative of 3 independent experiments. *$P < 0.05$ as compared to the control condition (Mann-Whitney U test).



Supplementary Figure 2. IFN-λ1 stimulation in combination with TLR7/8 ligation is able to activate both naïve and memory B cells

(A) PBMCs (n = 6) were stimulated 24 hours with IFN-λ or IFN-α at varying concentrations, with or without the combination of TLR7/8 agonist R848, and flow cytometric analysis was performed to determine the expression of the activation markers CD69 on $CD3^-/CD19^+$ B cells. (B) PBMCs (n = 6) were stimulated 3, 6, or 24 hours with IFN-λ or IFN-α, with or without the combination of TLR7/8 agonist R848, and flow cytometric analysis was performed to determine the expression of the activation markers CD69 on $CD3^-/CD19^+/CD27^{-/+}$ naïve and memory B cells. Data are shown as mean ± SEM and are representative of 3 independent experiments. *$P < 0.05$ as compared to the control condition (Mann-Whitney U test).



CHAPTER 4

IFN-Λ-MEDIATED IL-12 PRODUCTION IN MACROPHAGES INDUCES IFN-Γ PRODUCTION IN HUMAN NK CELLS

Rik A. de Groen[1], Arjan Boltjes[1], Jun Hou[1], Bi-Sheng Liu[1,2], Fiona McPhee[3], Jacques Friborg[3], Harry L.A. Janssen[1,4], and André Boonstra[1]

[1] Department of Gastroenterology and Hepatology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[2] Department of Rheumatology, Leiden University Medical Center, Leiden, the Netherlands
[3] Research and Development, Bristol-Myers Squibb, Wallingford, CT, USA
[4] Liver Clinic University Health Network, Division of Gastroenterology, University of Toronto, Canada

EUR J IMMUNOL. 2015 JAN;45(1):250-9.

ABSTRACT

With increasing interest in alternative options to interferon-alpha (IFN-α)-based treatments, IFN-λ has shown therapeutic promise in a variety of diseases. Although the antiviral activity of IFN-λ has been extensively studied, there is limited knowledge regarding the immunological functions of IFN-λ and how these differ from those of other classes of IFNs. In this study, we investigated the effects of IFN-λ on primary human natural killer (NK) cells, both in a direct and indirect capacity. We demonstrate that in contrast to IFN-α, IFN-λ is unable to directly stimulate NK cells, due to the absence of IFN-λ receptor chain 1 (IFN-λR1) on NK cells. However, IFN-λ, in combination with TLR4 challenge, is able to induce the production of select members of the IL-12 family of cytokines in monocyte-derived macrophages. We further show that through macrophage-mediated IL-12 production, IFN-λ is able to indirectly affect NK cells and ultimately induce IFN-γ production.

INTRODUCTION

Natural killer (NK) cells play an important role in the innate immune response, specifically in their ability to recognize and respond to stressed cells, including virus-infected, transformed, and damaged cells. Activated NK cells respond to these stress signals by excretion of cytotoxic factors, including perforin and granzymes, as well as cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor (TNF), that act as a first response to control the source of distress as well as to activate subsequent adaptive immune responses[1, 2]. NK cells express an array of activating receptors, such as C-type lectin-like receptors (e.g. NKG2D) and natural cytotoxicity receptors (NCRs), and inhibiting receptors, C-type lectin-like receptors (e.g. NKG2A) and killer cell immunoglobulin-like receptor (KIRs). It is currently believed that NK-cell activation or inhibition is governed by a balance of signaling by these receptors, and results after a critical threshold of activation signals exceeds inhibition[3].

NK-cell activation ensues after interacting with infected or distressed cells, shifting the balance of inhibitory and activating stimuli delivered via specific surface molecules. These include stress-induced surface markers, including altered MHC expression, as well as soluble factors, of which IL-12 and IL-18 are well-described and known triggers of NK-cell effector activity[1, 2, 4]. Various cell types, such as dendritic cells (DCs) and macrophages, are able to modulate the function of NK cells by changing the cytokine micro-environment and interacting with reciprocal surface molecules[5–8]. These interactions have been shown to play an important role in anti-tumor and anti-inflammatory responses in a myriad of diseases. In line with this, the activity of NK cells can be promoted by exposure to interferon-α (IFN-α), e.g. during interaction with IFN-producing activated plasmacytoid DCs leading to enhanced activation and cytolytic activity[9–11], and thereby promoting antiviral immunity.

In recent years, the type III family of IFNs, comprised of IFN-λ1, IFN-λ2, IFN-λ3, and IFN-λ4, has received increased attention, especially after the discovery of polymorphisms within its gene locus that were associated with spontaneous as well as therapy-induced clearance of hepatitis C virus (HCV)[12–15]. All members of the IFN-λ family use a specific receptor heterodimer, the IFN-λR1 and IL-10R2 complex, which structurally differs from the IFN-α receptor complex, but they trigger common JAK/STAT signaling cascades, albeit with different kinetics. For both types of IFN, phosphorylation of STAT1 and STAT2 has been described, which form complexes that bind promoter sequences in DNA ultimately resulting in the initiation of gene transcription[16, 17]. In contrast to the ubiquitously expressed IFN-α receptor, the distribution of the IFN-λ receptor is more limited. Murine models have shown that epithelial cells express the receptor complex and responded to IFN-λ stimulation by eliciting an antiviral response[18]. Outside of non-hematopoietic cells, specifically epithelial cells and hepatocytes, expression

of the IFN-λR1 chain in humans has been well-described on plasmacytoid DCs, fibroblasts[19, 20], and recently on macrophages[21].

We have previously demonstrated that monocyte-derived macrophages expressed a functional IFN-λ receptor complex, and that triggering of this receptor in combination with activation via specific toll-like receptors (TLRs) resulted in an enhancement of macrophage IL-12p40 production[21]. This was in contrast to IFN-α, which inhibited TLR-induced IL-12p40 production in monocyte-derived macrophages. IL-12p70 was, however, not detected in the supernatants of these stimulated macrophages, due to the absence of additional stimulatory signals, such as CD40 ligation or IFN-γ, required for the production of bioactive IL-12[22–24]. At present, it is unclear how triggering of similar signaling pathways by IFN-α and IFN-λ results in divergent biological activities. Besides macrophages, further functional differences have been described between IFN-α and IFN-λ in their modulation of hepatocytes, where IFN-λ has been shown to activate a more restricted subset of cells as well as induce sustained interferon-stimulated gene responses[25, 26].

To further define a role for IFN-λ in antiviral immune responses, the goal of this study was to determine whether IFN-λ could exert any immunomodulatory effects on NK cells, and if so, how that response compared to the action of type I IFNs. A previous study had described IFN-λ as being able to partially inhibit NK cell-derived IFN-γ production upon IL-12/15 stimulation[27, 28], but an effect of IFN-λ on NK cells could not be reproduced in an ensuing response to the original article[29]. To better understand the biological role of IFN-λ, we investigated the effects of IFN-λ on NK cells in both a direct and indirect capacity through its modulation of TLR-activated macrophages.

RESULTS

In contrast to IFN-αR1/2, IFN-λR1 is only expressed after monocyte to macrophage differentiation

The direct effects of IFN-α on NK cells have been well-described[10, 11], but the ability of IFN-λ to act on NK cells has received little attention. To address this issue, our first approach was to determine if human NK cells express a functional IFN-λ receptor. This was achieved by comparing the mRNA expression of IFN-α and IFN-λ receptors on primary NK cells and NK92 cells by quantitative PCR (qPCR). A HEK293 cell line stably transfected to express IFN-λ receptor mRNA as well as primary human hepatocytes, known to express the receptor and respond to IFN-λ[25, 26], were included as comparative positive controls, while the monocytic THP-1 cell line as well as primary human monocytes were included as negative controls. Although both IFN-αR1 and IFN-αR2 chains were ubiquitously expressed across all primary cells and cell lines (Figure 1A), IFN-λR1 mRNA expression was not detected in either primary NK or NK92 cells (Figure 1B). Monocytes

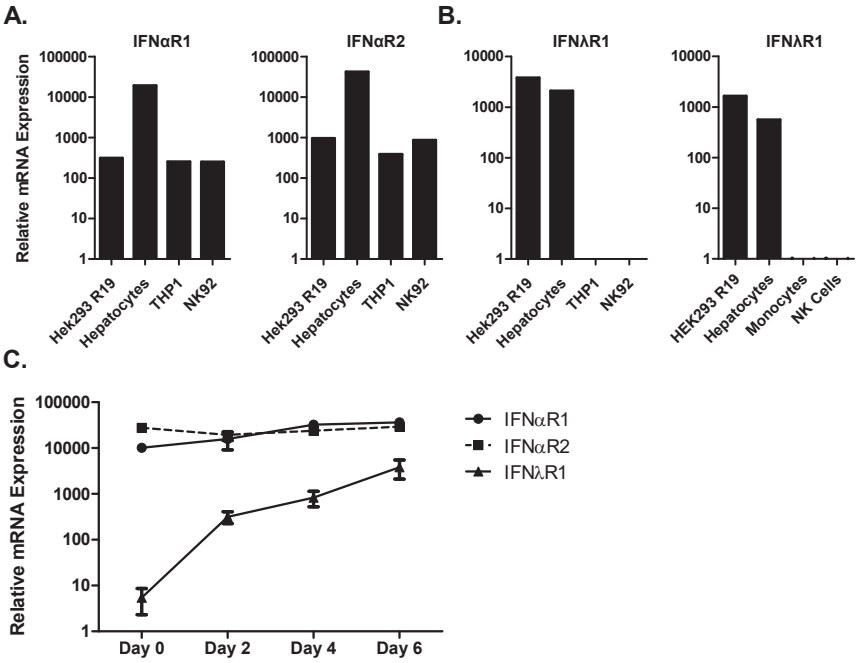


Figure 1. IFN-αR1/2 are ubiquitously expressed across both primary NK and monocytes and NK92/THP-1 cell lines, whereas IFN-λR1 is only expressed after monocyte to macrophage differentiation

(A and B) Expression of IFN-αR1, IFN-αR2, and IFN-λR1 genes in THP-1 and NK92 cell lines, and expression of IFN-λR1 genes in primary human monocytes and NK cells (n = 3 samples). The relative mRNA expression was measured by qPCR, using primary hepatocytes and HEK293 cells stably transfected to express IFN-λR1 (HEK293R19) as positive controls. (C) mRNA expression of IFN-αR1, IFN-αR2, and IFN-λR1 measured in monocytes (n = 6) at day 0, 2, 4, and 6 of culture with GM-CSF to induce macrophage differentiation was measured by qPCR. All expression levels are shown as the ΔΔCT relative to the housekeeping gene GAPDH. (A-C) Data are representative of 2 independent experiments, and (C) are shown as mean ± SEM (n = 6 samples).

were also devoid of IFN-λR1 expression; however, differentiation of monocytes to macrophages by culture with granulocyte macrophage colony-stimulating factor (GM-CSF) resulted in an induction and continuous upregulation of IFN-λR1 over a 6 day period (Figure 1C). IFN-αR1 and IFN-αR2, both already highly expressed on monocytes, remained relatively unaltered across the differentiation process.

IFN-α, but not IFN-λ1, is able to directly stimulate and enhance effector function in NK cells

To further investigate NK cell responsiveness to type III IFNs, isolated primary NK cells were incubated with either IFN-α or IFN-λ1 overnight and analyzed for their expression of various activation markers and inhibition/activation receptors,

including CD69, a marker of lymphoid cell activation, and C-type lectin transmembrane receptors NKG2A and NKG2D. The percentage of CD69 expressing NK cells was increased by almost sevenfold in IFN-α cultures (76.3%) compared to those exposed to medium alone (11.3%) or IFN-λ1 (13.5%) (Figure 2A), while expression of inhibitory receptor NKG2A and the activating receptor NKG2D remained unaffected in all conditions (Figure 2A and data not shown). The cytolytic capacity of IFN-stimulated NK cells was assessed by measuring the degranulation marker CD107a after coculture with the target cell line K562, and by mRNA expression of protease granzyme B (GZMB). NK cells exposed to IFN-α during the 5 hour coculture period with K562 cells expressed higher levels of CD107a (32.2%) while coculture with IFN-λ1 (21.1%) showed no difference from the medium condition (19.8%) (Figure 2B). Stimulation with IFN-α, in combination with IL-18, was also able to upregulate GZMB expression tenfold after 5 hours,

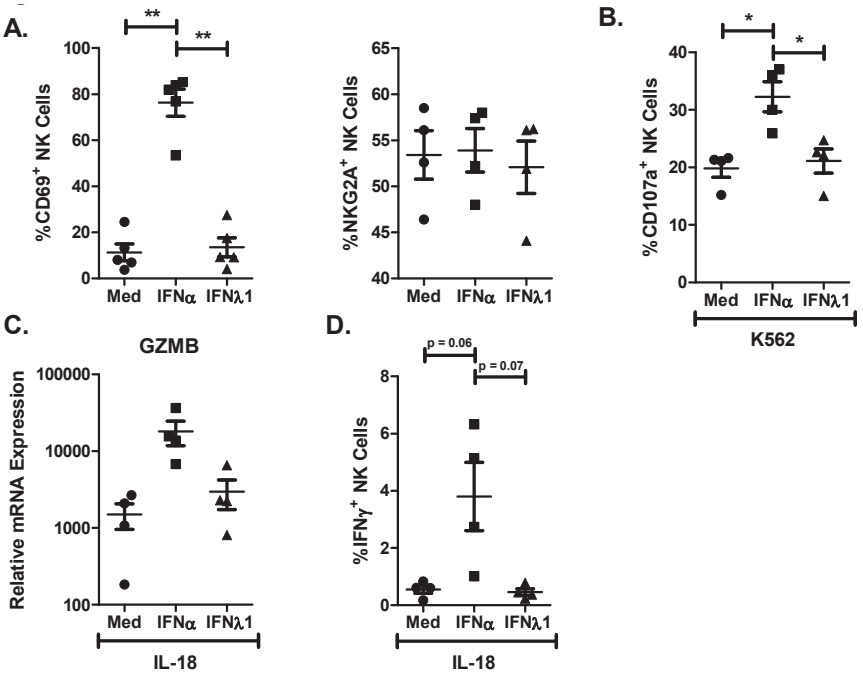


Figure 2. IFN-α, but not IFN-λ1, directly stimulates and enhances effector function in NK cells
(A) Surface marker and intracellular flow cytometric analysis of CD69 (n = 5 samples) and NGK2A (n = 4) in isolated primary NK cells, stimulated overnight with IFN-α or IFN-λ1. (B) The cytolytic capacity of IFN stimulated NK cells was assessed by flow cytometry analysis of degranulation marker CD107a after 5 hours of coculture with target cell line K562 (n = 4). (C) mRNA expression of protease granzyme B (GZMB) (n = 4) in NK cells, stimulated with IFN-α or IFN-λ1 in combination with IL-18, measured by qPCR. Expression is shown as the ΔΔCT relative to housekeeping gene GAPDH. (D) Intracellular flow cytometry analysis of IFN-γ (n = 4) in isolated primary NK cells, stimulated overnight with IFN-α or IFN-λ1 in combination with IL-18. Data are shown as mean ± SEM and are representative of 2 independent experiments. **P* < 0.05. ***P* < 0.01; Mann-Whitney U test.

whereas IFN-λ1 again remained ineffective (Figure 2C). Lastly, isolated primary NK cells were incubated with either IFN-α or IFN-λ1, in addition to IL-18, overnight and analyzed by intracellular flow cytometry for production of IFN-γ (Figure 2D) and TNF (data not shown). In contrast to IFN-α, IFN-λ1 was unable to directly increase the frequency of primary NK cells producing IFN-γ, but not TNF, upon IL-18 stimulation. This highlights a direct action of IFN-α on NK cells, resulting in activation, cytotoxicity, and cytokine production, that does not exist for IFN-λs, most likely due to the absence of IFN-λ receptor expression.

TLR-mediated IL-12 family expression is differentially modulated by IFN-λ1 and IFN-α in macrophages

The interactions between NK cells and other innate immune cells have been well-documented in various diseases, with implications for anti-tumor and anti-inflammatory responses[5–8]. Although we determined that IFN-λ1 is unable to directly affect NK cells, an indirect effect was still feasible, exerted through cross-talk between macrophages and NK cells in a cytokine-dependent manner. First, monocyte derived-macrophages, primed with IFN-α or IFN-λ1, were further challenged with TLR4 agonist LPS for an additional 4 hours for gene expression analysis or 24 hours for immunoassay analysis of proinflammatory cytokines. To determine to what extent IFN-λ1 stimulation regulates macrophage IL-12 related cytokine production, qPCR for various subunits of the IL-12 family were performed. As shown in Figure 3A, increased expression of IL12B (encoding IL-12p40), IL23A (IL-23p19), and EBI3 were observed in IFN-λ1/LPS-stimulated macrophages compared to the IFN-α/LPS conditions. IL27A (IL-27p28) mRNA was upregulated by incubation of both IFN-α/LPS and IFN-λ1/LPS, whereas IL12A (IL-12p35) mRNA expression was undetectable in all conditions (data not shown).

To determine if the mRNA levels measured correlated with protein production, cytokine levels of IL-12 family members were measured by ELISA in the supernatants of stimulated macrophages. Increased levels of IL-12p40 and IL-23 were detected in the supernatants of IFN-λ1/LPS-stimulated cells, in contrast to IFN-α/LPS stimulation (Figure 3B and C). IL-12p70 and IL-27 were not detected in any condition. These undetectable levels of IL-12p70 can be explained by the lack IL-12p35 mRNA expression previously seen by qPCR. Bioactive IL-12 was produced by supplementation of IFN-γ to the TLR challenge of macrophages after the initial priming with IFN-λ1, but not after IFN-α pretreatment (Figure 3D). Inter-culture variability was observed in levels of macrophage IL-12p40 production, but the trend of increased production after IFN-λ1 preincubation was seen in all cultures, while IFN-α response was more heterogeneous (Figure 3C).

Further multiplex analysis of macrophage-associated cytokines and chemokines showed significantly elevated levels of TNF, GM-CSF, and IP-10 in the supernatants of macrophages stimulated with IFN-λ1 compared to control and

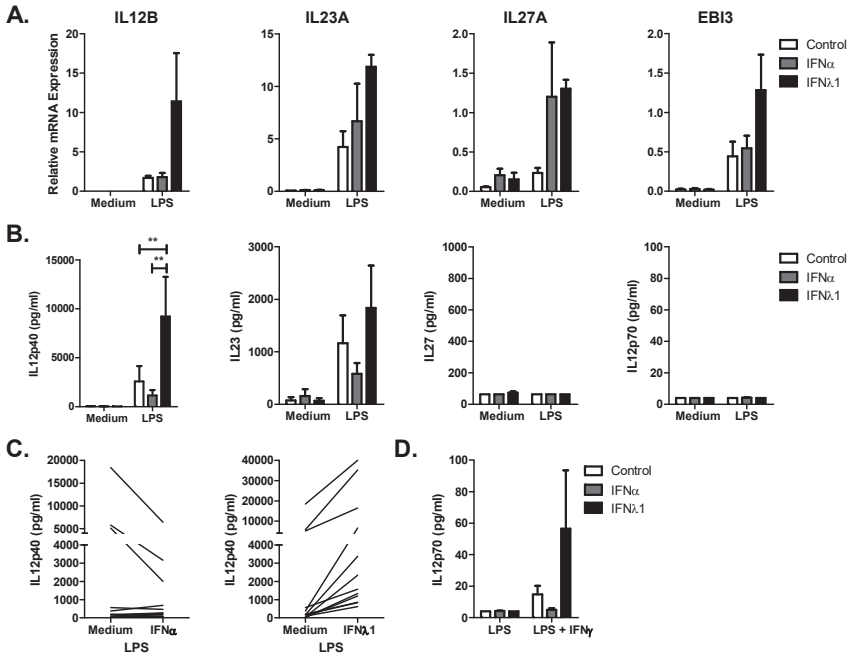


Figure 3. TLR-mediated IL-12 family cytokine expression is differentially modulated by IFN-λ1 and IFN-α in macrophages

(A) Levels of gene expression (n = 3 samples) and (B) protein (n = 13) of the IL-12 family members (IL-12p40, IL-12p70, IL-23, and IL-27) in macrophages stimulated with IFN-α or IFN-λ1 for 5 hours and then further challenged with LPS. mRNA expression levels were measured by qPCR after an additional 4 hours of TLR challenge, and protein levels by ELISA after an additional 24 hours. All gene expression is shown as the ΔΔCT relative to housekeeping gene GAPDH. (C) Individual results for IL-12p40 of IFN/LPS-stimulated macrophages (n = 13). (D) Bioactive IL-12p70 levels were measured by ELISA in the supernatants of macrophages preincubated of IFN-α or IFN-λ1 for 5 hours, followed by an additional 24 hours of stimulation with LPS and IFN-γ (n = 8). (A, B, and D) Data are shown as mean + SEM and (A-D) are representative of 3 independent experiments, and $*P < 0.05$. $**P < 0.01$; Mann-Whitney U test.

IFN-α conditions (Supporting Information Figure 1). Overall, differences can be observed between IFN-α and IFN-λ1 in their modulation of TLR-induced cytokine production by macrophages, with a specific emphasis on multiple members of the IL-12 family.

IFN-λ1-mediated IL-12 production by macrophages induces IFN-γ production by NK and NK92 cells

To test the effects of IFN-λ on cross-talk between macrophages and NK/NK92 cells, supernatants from macrophages incubated with IFN-α or IFN-λ1 in combination with LPS and IFN-γ were used to stimulate primary NK cells and NK92 cells overnight supplemented with IL-18. The NK cell/NK92 effector function was

then determined by intracellular FACS analysis of IFN-γ and TNF production in the respective conditions. Supernatants from IFN-λ1/LPS-stimulated macrophages were able to increase the percentage of IFN-γ-producing cells, but not TNF-producing cells (data not shown) when compared with 3 NK cell donors treated with supernatants from medium/LPS- stimulated macrophages (Figure 4A and B). The same was true for NK92 cell cultures, where a 2.5-fold increase in IFN-γ positive cells was observed compared to NK92 cell cultures treated with supernatants from both medium/LPS and IFN-α/LPS stimulated macrophages (Figure 4C and D). It should be noted that increased IFN-γ production was also

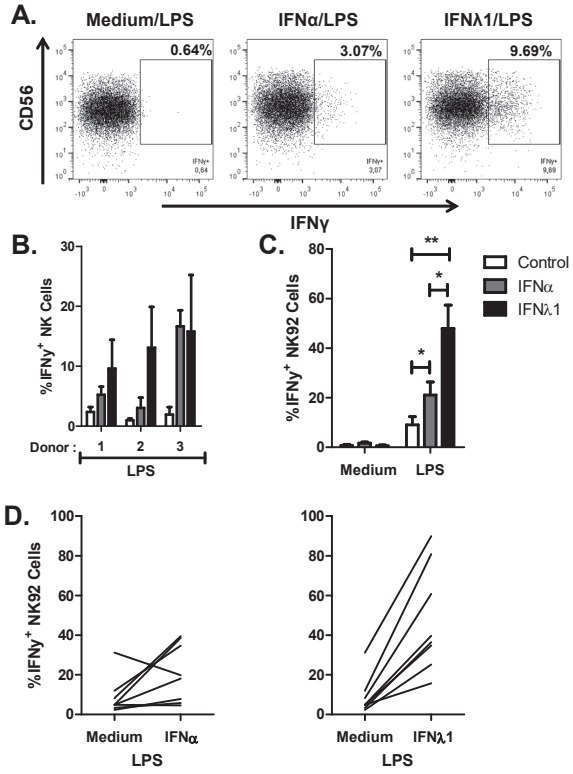


Figure 4. IFN-λ1 mediated IL-12 production of macrophages is able to induce effector function in NK and NK92 cells

Supernatant from macrophages treated with IFN-α or IFN-λ1 in combination with LPS and IFN-γ were used for overnight stimulation of (A and B) isolated primary NK cells from three healthy donors and (C and D) NK92 cells. All supernatants were supplemented with IL-18 during overnight stimulation. (A) Intracellular IFN-γ levels were measured using flow cytometry. Plots are representative of 5 and 8 independent experiments respectively. (B and C) Data are shown as mean ± SEM ((B) n = 5 or (C) n = 8 samples) and arerepresentative of 2 independent experiments. (D) Individual results for supernatants of multiple donors for macrophage-mediated IFN-γ production in NK92 cells. Data shown are representative of 2 independent experiments. **P* < 0.05. ***P* < 0.01; Mann-Whitney U test.

seen with the IFN-α pretreated supernatants, but this can partially be attributed to the direct action of IFN-α on NK and NK92 cells.

IFN-γ production in NK92 cells is IL-12 dependent in IFN-λ1/LPS-stimulated macrophages

To determine the mechanism of action for induction of NK92 effector function by IFN-stimulated macrophages, various blocking and neutralization assays were performed. Neutralization of IL-12p40 in IFN-λ1/LPS-stimulated macrophages abrogated IL-12-mediated IFN-γ production in NK92 cells while neutralization of IL-12p40 in IFN-α/LPS-stimulated macrophages partially abrogated IL-12-mediated IFN-γ, but not TNF, production in NK92 cells (Figure 5A-C). Blocking of the

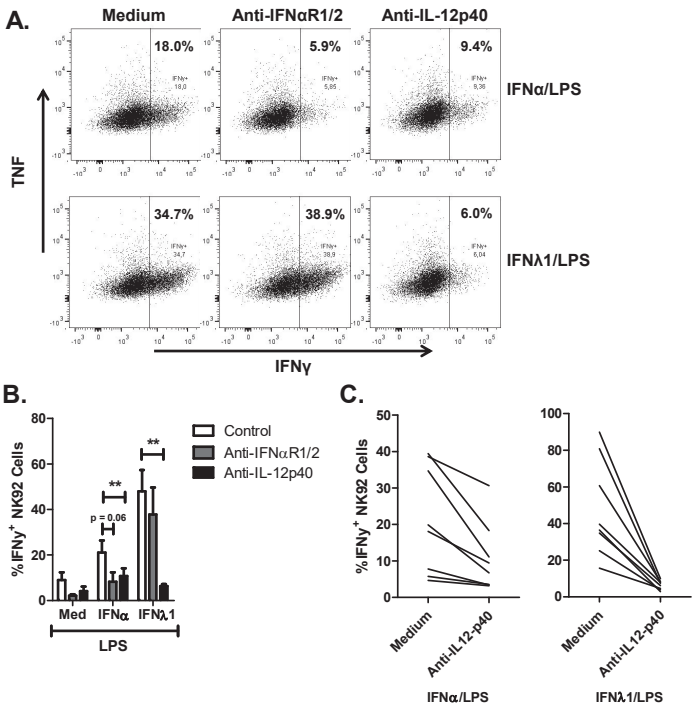


Figure 5. Macrophage-mediated IFN-γ production in NK92 cells is IL-12-dependent in IFN-λ1/LPS but not in IFN-α/LPS-stimulated macrophages

NK92 cells were cultured with the supernatants of IFN and LPS-stimulated macrophages in the presence of neutralizing/blocking antibodies for IL-12p40 and IFN-αR1/2 (n = 8 samples). All supernatants were supplemented with IL-18 during overnight stimulation. (A) Intracellular IFN-γ and TNF was measured using flow cytometry. Plots are representative of 2 independent experiments. (B) The percentage of IFN-γ+ cells in NK92 cells was measured by flow cytometry. Data are shown as mean ± SEM (n = 8 samples) and are representative of 2 independent experiments. (C) Individual results for IL-12p40 neutralization in supernatants from all 8 samples for macrophage-mediated IFN-γ production in NK92 cells. Data shown are representative of 2 independent experiments. **P* < 0.05. ***P* < 0.01; Mann-Whitney U test.

IFN-α receptor in macrophages stimulated with IFN-α/LPS showed a strong, but not significant, trend in reduction in IFN-γ production in NK92 cells, however no effect was observed after stimulation with IFN-λ1/LPS. Due to the heterodimeric nature of the IL-12 family, we were unable to exclude an effect of IL-23 on IFN-γ production of NK cells, and therefore performed identical assays using IL-23p19 neutralizing antibodies. Again IL-12p40 neutralization resulted in an almost complete diminishment of IFN-γ producing NK92 cells, while IL-23p19 neutralization had no effect (Supporting Information Figure 2). These results confirm the direct action of IFN-α on NK cell effector function, and demonstrate that IFN-λ1 acts indirectly on NK cells through macrophage-derived IL-12p40 production.

DISCUSSION

The focus of this study was to investigate the ability of IFN-λ1 to modulate the activity of NK cells in both a direct and an indirect capacity. We demonstrate that unlike IFN-α, IFN-λ1 is unable to directly activate and promote effector function in NK cells, presumably due to the lack of cellular expression of the complete type III IFN receptor complex. However, IFN-λ1 priming, in combination with TLR4 activation, is able to induce select members of the IL-12 family of cytokines in monocyte-derived macrophages. We further show that IFN-λ1, in combination with IFN-γ, is able to indirectly affect NK cells through macrophage-mediated IL-12 production, ultimately inducing IFN-γ production.

As a first step, we demonstrate that NK cells do not express the IFN-λR1 chain, and that consequently IFN-λ1 is unable to directly act on this cell population. This is in contrast to previous reports suggesting that type III IFNs have an inhibitory effect on IFN-γ production upon IL-12/IL-15 stimulation[27, 28]. Our study not only investigated the effects of type I and III IFNs on cytokine production, but also on NK cell activation and cytotoxicity. We showed that IFN-α is able to activate as well as promote cytolytic action and IFN-γ production by NK cells, whereas IFN-λ1 was unable to alter any of these parameters. These findings are corroborated in a response to Dring et al. that questioned their original findings, also reporting the absence of IFN-λR1 receptor expression in primary NK cells and the inability of IFN-λ to induce IFN-γ production or degranulation in any capacity[29, 30]. Combined, these results convincingly show that IFN-λ lacks the ability to directly activate and affect multiple facets of NK cell effector function.

We do, however, propose for the first time an indirect link between IFN-λ1 and NK cells, mediated through the stimulation of macrophages. This interaction was unique to IFN-λ1, due to differences in immunomodulatory properties between IFN-α and IFN-λ1, specifically in their regulation of various subunits of the IL-12 family of cytokines (IL12B, IL23A, and EBI3) in macrophages. In contrast to IFN-λ1, which promoted the production of specific IL-12-related cytokines (IL-12

and IL-23), IFN-α actually reduced macrophage-facilitated IL-12 production. Through the blocking of IFN-αR1/2 on NK cells and neutralizing IL-12 in supernatants from IFN-primed macrophages, we were able to show that enhancement of NK-cell derived IFN-γ production by IFN-α was mainly mediated by a direct interaction with NK cells, and partially affected by IL-12 neutralization. IFN-λ1, on the other hand, was dependent on IL-12, with almost complete abrogation of IFN-γ-producing NK cells after IL-12 neutralization. Despite having different modes of action, both the direct effects of IFN-α and indirect effects of IFN-λ1 were found to be independent of cell-to-cell contact since soluble factors in IFN/TLR-challenged macrophage supernatants, supplemented with IL-18, were sufficient to induce IFN-γ production in NK cells. It is of note, that IFN-γ stimulation of macrophages was needed for the production of bioactive IL-12, potentially leading to a positive feedback loop between NK cells and macrophages of IFN-γ and IL-12 production. Previous publications have also described a role for IL-12-related cytokines in macrophage-NK cell interactions. Specifically, macrophage-derived expression of IL-23 and IL-27 induced the production of IFN-γ and GM-CSF in NK cells[31–33]. Although the production of IL-27 was monitored in IFN-λ-stimulated macrophages, the effects on NK cell function was not explored in this study.

In contrast to the ubiquitous expression of the IFN-αR1/2 subunits across various cell populations, resting monocytes do not express IFN-λR1 until after the initiation of macrophage differentiation via culture with GM-CSF, resulting in an induction and constant upregulation of receptor expression. The expression of the IFN-α receptor on NK cells and the ability of IFN-α to directly activate these cells, promoting degranulation, cytotoxicity, and IFN-γ production, are distinct from the effects of IFN-λ. These features of IFN-α activation make it an important mediator in the induction of an immediate immune response upon challenge in the peripheral as well as the tissue compartments. In contrast, the restricted expression of IFN-λR1 prevented a direct action of IFN-λ on both NK cells and monocytes, with only responses observed on differentiated macrophages. This suggests that IFN-λ might play a more prominent role in tissue-specific responses as compared to the periphery, in line with other studies describing that IFN-λ1 has a more delayed and prolonged effect through activation of specific tissue-resident cells, specifically epithelial cells and hepatocytes[25, 26]. TLR-dependent cross-talk between NK cells and macrophages has already been described in tissue-specific interactions in the liver. TLR-activated liver-resident macrophages have been shown to exert effector function in NK cells, specifically by inducing their activation and IFN-γ production[34]. The ability of IFN-λ1 to modulate TLR-induced cross-talk between macrophages and NK cells could have implications in the enhancement of IFN-γ production by liver NK cells, a critical factor in the inhibition of HCV replication and in the response to HBV infection[35–37]. The delayed activation of NK cells by IFN-λ, mediated through macrophage IL-12 production, could explain the observed differences in biological functions between the type I

and type III IFNs in inflammatory responses, but also by differences in kinetics or sustainability of the response. It should be noted that a similar kinetic on innate immune response activation by type III IFNs has been previously described for DCs and hepatocytes[26, 30, 38].

Collectively, this study provides the first example of an indirect regulatory effect unique to IFN-λ1, mediated through macrophage cytokine production and independent of cell-to-cell contact. This is distinct from IFN-α, which directly activates NK cells, and can inhibit macrophage IL-12 production. Further studies of both the antiviral and immunoregulatory effects of IFN-λ1 are warranted to understand the basic biology of type III IFNs and their activities as therapeutic agents.

MATERIALS AND METHODS

Cell culture and isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Sanquin Blood Supply) using density gradient centrifugation (Ficoll-Paque, GE Healthcare). Monocytes and NK cells were then purified from the PBMC fraction using magnetic labeled CD14 micro-beads (Miltenyi Biotec) and human NK Cell Isolation Kit (Miltenyi Biotec) according to the protocol provided by the manufacturer. The purity of monocytes and NK cells isolated and used in this study always exceeded 95%.

Macrophages were generated from the aforementioned purified monocytes by culturing with 20 ng/ml GM-CSF (Leukine® (sargramostim)) in 6-well plates (Costar) at a density of 0.75 x 106 cells/ml in RPMI1640 medium (Lonza) supplemented with 10% fetal calf serum (Sigma), penicillin/streptomycin (Gibco), L-glutamine (Lonza), and HEPES (Lonza). The medium was partially refreshed at days 2 and 5, and the monocyte-derived macrophages were harvested at day 6 for use in various assays.

HEK293 cells that were stably transfected to express the IFN-λ receptor (HEK293 R19) were cultured using RPMI1640 medium supplemented with 10% fetal calf serum. THP-1, NK92, and K562 cell lines were cultured using the recommended conditions by the ATCC.

Stimulation of monocyte-derived macrophages

Monocyte-derived macrophages were cultured in X-Vivo medium (Lonza) with 10 ng/ml IFN-α-2b (Intron A, Merck) or 100 ng/ml IFN-λ1 (Bristol-Myers Squibb) for 5 hours and then further challenged with the TLR4 agonist lipopolysaccharide (100 ng/ml; LPS-SM Ultrapure, InvivoGen) for an additional 6 or 24 hours at a cell density of 1 x 106 cells/ml. Cells were harvested at 4 hours for mRNA analysis, and supernatants were harvested at 24 hours for immunosorbent and

further assays. In a fraction of the assays, 10 ng/ml IFN-γ (Miltenyi Biotec) was supplemented along with the LPS addition for the production of bioactive IL-12 for later stimulation of NK cells.

Stimulation and intracellular cytokine analysis of NK/NK92 cells

Isolated primary NK cells and NK92 cells were stimulated with 10 ng/ml IFN-α-2b (Intron A, Merck) or 100 ng/ml IFN-λ1 (Bristol-Myers Squibb) alone or in combination with 100 ng/ml IL-18 (R&D Systems) for 5 hours for quantitative PCR or overnight for surface marker expression and intracellular cytokine production. Cellular activation and surface marker expression was measured using flowcytometric analysis with anti-CD69-Pacific Blue (FN50, Biolegend), anti-NKG2A-PE (Z119, Beckman Coulter), and anti-NKG2D-PerCP (ID11, BD Pharmingen).

The aforementioned supernatants of monocyte-derived macrophages were diluted in a 1:1 ratio with RPMI1640 containing 10% FCS and used to stimulate primary NK cells and NK92 cells for 24 hours with the supplementation of IL-18 (100 ng/ml). Antibodies against IFN-αR1/2 (MRHAR-2, Millipore), IL-12p40 (C8.6, eBioscience) and IL-23p19 (HNU2319, eBioscience) were added for blocking and neutralizing purposes, respectively.

For analysis of cytokine production in NK cells, 10 µg/ml brefeldin A (Sigma) was added after 18 hours and cells were incubated for an additional 3 hours. Samples were then fixed with 2% formaldehyde, and permeabilized for intracellular staining with anti-IFN-γ-PE-Cy7 (eBioscience) and anti-TNF-PE (BD Pharmingen). Cytokine-producing cells were detected by flow cytometry (FACS Canto II, BD) and analyzed using FlowJo (Tree Star Incorporated).

NK cell CD107a degranulation

Isolated NK cells (0.1 x 106 cells/200 µl) were seeded with K562 cells in a 96 well plate in a 1:1 effector to target ratio. IFN-α-2b (10 ng/ml; Intron A, Merck) or recombinant IFN-λ1 (100 ng/ml; Bristol-Myers Squibb) as well as anti-CD107a-PE (H4A3, BD Pharmingen) were added to the culture and after 90 minutes, GolgiStop (BD) was added, followed by incubation for an additional 3.5 hours. Degranulated cells were detected by flow cytometry (FACS Canto II, BD) and analyzed using FlowJo (Tree Star Incorporated).

Immunoassay for detection of cytokines in supernatants

The concentrations of various cytokines were measured in the supernatants of stimulated macrophages by the use of sandwich ELISAs specific for IL-12p40 (clones C8.6 and C8.3, Biolegend), IL-12p70, IL-23, IL-27, and TNF (Ready-Set-Go Kits, eBioscience) with a sensitivity of 30 pg/ml, 4 pg/ml, 15 pg/ml, 64 pg/

ml, and 4 pg/ml, respectively. Simplex bead sets for IL-1α, IL-1β, IL-1RA, IL-6, IL-12p40, IL-18, IFN-β, GM-CSF, TNF, and IP-10 (eBioscience) were used to measure the concentrations of cytokines and chemokines in supernatants with sensitivity ranges of 1–630 pg/ml, 3–3 000 pg/ml, 38–150000 pg/ml, 10–5000 pg/ml, 5–1350 pg/ml, 11–12700 pg/ml, 11–2500 pg/ml, 14–3 600 pg/ml, 4–3000 pg/ml, and 3–650 pg/ml respectively.

Gene expression quantification of IFN-αR1/2, IFN-λR1, GZMB, and IL-12 family subunits

Total RNA was isolated from HEK293 R19, THP-1, and NK92 cell lines as well as from primary human hepatocytes (isolated from healthy donors livers), primary NK cells, monocytes, and monocyte-derived macrophages using the RNeasy kit (Qiagen) and cDNA was prepared using the Primescript cDNA synthesis kit (Takara) from 500 ng RNA. All real-time PCR reactions were performed using a MyIQ5 detection system (Bio-Rad) and relative mRNA expression levels were calculated using the housekeeping gene GAPDH. The following primer/probe sets (Applied Biosystems) or forward/reverse SYBR Green primer sequences were used for the genes of interest:

GAPDH (Hs00266705_g1), IFN-αR1 (Hs01066115_m1),
IFN-αR2 (Hs01022060_m1), IFN-λR1 (Hs00417120_m1),
GZMB (Hs01554355_m1)

IL-12p35:	Fw: 5'-CCACTCCAGACCCAGGAATG-3',
	Rv: 5'-GACGGCCCTCAGCAGGT-3'
IL-12p40:	Fw: 5'-ACGGACAAGACCTCAGCCAC-3',
	Rv: 5'-GGGCCCGCACGCTAA-3'
IL-23p19:	Fw: 5'-GAGCCTTCTCTGCTCCCTGAT-3',
	Rv: 5'-AGTTGGCTGAGGCCCAGTAG-3'
IL-27p28:	Fw: 5'-GCGGAATCTCACCTGCCA-3',
	Rv: 5'-GGAAACATCAGGGAGCTGCTC-3'
EBI-3:	Fw: 5'-CCGAGCCAGGTCCTACGTCC-3',
	Rv: 5'-CCAGTCACTCAGTTCCCCGT-3'

STATISTICAL ANALYSIS

Data is expressed as the mean value ± standard error of the mean (SEM) unless indicated otherwise. Data was analyzed with Prism 5.0 software (GraphPad) using the Mann-Whitney U test to compare variables between independent groups and the Spearman rank correlation coefficient test for nonparametric correlations.

In all analyses, a 2-tailed P value of less than 0.05 (confidence interval of 95%) was considered statistically significant.

ACKNOWLEDGMENTS

This study was supported by the Virgo consortium, funded by the Dutch government project number FES0908, and by the Netherlands Genomics Initiative (NGI) project number 050–060–452; and further supported by funding of Bristol-Myers Squibb.

CONFLICTS OF INTEREST DISCLOSURE

This study was financially supported by Bristol-Myers Squibb (BMS), and F. McPhee and J Friborg. are employees of BMS. This, however, has not influenced the conclusions or the data presented in the current article.

REFERENCES

1 Vivier, E., Raulet, D. H., Moretta, A., Caligiuri, M. A., Zitvogel, L., Lanier, L. L., Yokoyama, W. M. and Ugolini, S., Innate or adaptive immunity? The example of natural killer cells. Science 2011. 331: 44-49.

2 Lanier, L. L., NK cell recognition. Annu Rev Immunol 2005. 23: 225-274.

3 Mondelli, M. U., Varchetta, S. and Oliviero, B., Natural killer cells in viral hepatitis: facts and controversies. Eur J Clin Invest 2010. 40: 851-863.

4 Long, E. O., Kim, H. S., Liu, D., Peterson, M. E. and Rajagopalan, S., Controlling natural killer cell responses: integration of signals for activation and inhibition. Annu Rev Immunol 2013. 31: 227-258.

5 Nedvetzki, S., Sowinski, S., Eagle, R. A., Harris, J., Vely, F., Pende, D., Trowsdale, J., Vivier, E., Gordon, S. and Davis, D. M., Reciprocal regulation of human natural killer cells and macrophages associated with distinct immune synapses. Blood 2007. 109: 3776-3785.

6 Lapaque, N., Walzer, T., Meresse, S., Vivier, E. and Trowsdale, J., Interactions between human NK cells and macrophages in response to Salmonella infection. J Immunol 2009. 182: 4339-4348.

7 Tjwa, E. T., van Oord, G. W., Biesta, P. J., Boonstra, A., Janssen, H. L. and Woltman, A. M., Restoration of TLR3-activated myeloid dendritic cell activity leads to improved natural killer cell function in chronic hepatitis B virus infection. J Virol 2012. 86: 4102-4109.

8 Klezovich-Benard, M., Corre, J. P., Jusforgues-Saklani, H., Fiole, D., Burjek, N., Tournier, J. N. and Goossens, P. L., Mechanisms of NK cell-macrophage Bacillus anthracis crosstalk: a balance between stimulation by spores and differential disruption by toxins. PLoS Pathog 2012. 8: e1002481.

9 Trinchieri, G. and Santoli, D., Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis. J Exp Med 1978. 147: 1314-1333.

10 Trinchieri, G., Santoli, D., Dee, R. R. and Knowles, B. B., Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Identification of the anti-viral activity as interferon and characterization of the human effector lymphocyte subpopulation. J Exp Med 1978. 147: 1299-1313.

11 Gerosa, F., Gobbi, A., Zorzi, P., Burg, S., Briere, F., Carra, G. and Trinchieri, G., The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. J Immunol 2005. 174: 727-734.

12 Suppiah, V., Moldovan, M., Ahlenstiel, G., Berg, T., Weltman, M., Abate, M. L., Bassendine, M., Spengler, U., Dore, G. J., Powell, E., Riordan, S., Sheridan, D., Smedile, A., Fragomeli, V., Muller, T., Bahlo, M., Stewart, G. J., Booth, D. R. and George, J., IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 2009. 41: 1100-1104.

13 Tanaka, Y., Nishida, N., Sugiyama, M., Kurosaki, M., Matsuura, K., Sakamoto, N., Nakagawa, M., Korenaga, M., Hino, K., Hige, S., Ito, Y., Mita, E., Tanaka, E., Mochida, S., Murawaki, Y., Honda, M., Sakai, A., Hiasa, Y., Nishiguchi, S., Koike, A., Sakaida, I., Imamura, M., Ito, K., Yano, K., Masaki, N., Sugauchi, F., Izumi, N., Tokunaga, K.

and Mizokami, M., Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009. 41: 1105-1109.

14 Thomas, D. L., Thio, C. L., Martin, M. P., Qi, Y., Ge, D., O'Huigin, C., Kidd, J., Kidd, K., Khakoo, S. I., Alexander, G., Goedert, J. J., Kirk, G. D., Donfield, S. M., Rosen, H. R., Tobler, L. H., Busch, M. P., McHutchison, J. G., Goldstein, D. B. and Carrington, M., Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 2009. 461: 798-801.

15 Ge, D., Fellay, J., Thompson, A. J., Simon, J. S., Shianna, K. V., Urban, T. J., Heinzen, E. L., Qiu, P., Bertelsen, A. H., Muir, A. J., Sulkowski, M., McHutchison, J. G. and Goldstein, D. B., Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009. 461: 399-401.

16 Donnelly, R. P. and Kotenko, S. V., Interferon-lambda: a new addition to an old family. J Interferon Cytokine Res 2010. 30: 555-564.

17 Rauch, I., Muller, M. and Decker, T., The regulation of inflammation by interferons and their STATs. JAKSTAT 2013. 2: e23820.

18 Ank, N., Iversen, M. B., Bartholdy, C., Staeheli, P., Hartmann, R., Jensen, U. B., Dagnaes-Hansen, F., Thomsen, A. R., Chen, Z., Haugen, H., Klucher, K. and Paludan, S. R., An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. J Immunol 2008. 180: 2474-2485.

19 Megjugorac, N. J., Gallagher, G. E. and Gallagher, G., Modulation of human plasmacytoid DC function by IFN-lambda1 (IL-29). J Leukoc Biol 2009. 86: 1359-1363.

20 Xu, L., Feng, X., Tan, W., Gu, W., Guo, D., Zhang, M. and Wang, F., IL-29 enhances Toll-like receptor-mediated IL-6 and IL-8 production by the synovial fibroblasts from rheumatoid arthritis patients. Arthritis Res Ther 2013. 15: R170.

21 Liu, B. S., Janssen, H. L. and Boonstra, A., IL-29 and IFNalpha differ in their ability to modulate IL-12 production by TLR-activated human macrophages and exhibit differential regulation of the IFNgamma receptor expression. Blood 2011. 117: 2385-2395.

22 Mosser, D. M. and Edwards, J. P., Exploring the full spectrum of macrophage activation. Nat Rev Immunol 2008. 8: 958-969.

23 Boonstra, A., Rajsbaum, R., Holman, M., Marques, R., Asselin-Paturel, C., Pereira, J. P., Bates, E. E., Akira, S., Vieira, P., Liu, Y. J., Trinchieri, G. and O'Garra, A., Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals. J Immunol 2006. 177: 7551-7558.

24 Trinchieri, G., Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 2003. 3: 133-146.

25 Jilg, N., Lin, W., Hong, J., Schaefer, E. A., Wolski, D., Meixong, J., Goto, K., Brisac, C., Chusri, P., Fusco, D. N., Chevaliez, S., Luther, J., Kumthip, K., Urban, T. J., Peng, L. F., Lauer, G. M. and Chung, R. T., Kinetic differences in the induction of interferon stimulated genes by interferon-alpha and interleukin 28B are altered by infection with hepatitis C virus. Hepatology 2014. 59: 1250-1261.

26 Olagnier, D. and Hiscott, J., Type I and type III interferon-induced immune response: It's a matter of kinetics and magnitude. Hepatology 2014. 59: 1225-1228.

27 Dring, M. M., Morrison, M. H., McSharry, B. P., Guinan, K. J., Hagan, R., Irish, H. C. V. R. C., O'Farrelly, C. and Gardiner, C. M., Innate immune genes synergize to predict increased risk of chronic disease in hepatitis C virus infection. Proc Natl Acad Sci U S A 2011. 108: 5736-5741.

28 Gardiner, C. M., Morrison, M. H. and Dring, M. M., Reply to Krämer et al.: Human natural killer (NK) cell inhibition by IL28A. PNAS 2011. 108: E521–E522.

29 Kramer, B., Eisenhardt, M., Glassner, A., Korner, C., Sauerbruch, T., Spengler, U. and Nattermann, J., Do lambda-IFNs IL28A and IL28B act on human natural killer cells? Proc Natl Acad Sci U S A 2011. 108: E519-520; author reply E521-512.

30 Yin, Z., Dai, J., Deng, J., Sheikh, F., Natalia, M., Shih, T., Lewis-Antes, A., Amrute, S. B., Garrigues, U., Doyle, S., Donnelly, R. P., Kotenko, S. V. and Fitzgerald-Bocarsly, P., Type III IFNs are produced by and stimulate human plasmacytoid dendritic cells. J Immunol 2012. 189: 2735-2745.

31 van de Wetering, D., de Paus, R. A., van Dissel, J. T. and van de Vosse, E., Salmonella induced IL-23 and IL-1beta allow for IL-12 production by monocytes and Mphi1 through induction of IFN-gamma in CD56 NK/NK-like T cells. PLoS One 2009. 4: e8396.

32 Laroni, A., Gandhi, R., Beynon, V. and Weiner, H. L., IL-27 imparts immunoregulatory function to human NK cell subsets. PLoS One 2011. 6: e26173.

33 Kastelein, R. A., Hunter, C. A. and Cua, D. J., Discovery and biology of IL-23 and IL-27: related but functionally distinct regulators of inflammation. Annu Rev Immunol 2007. 25: 221-242.

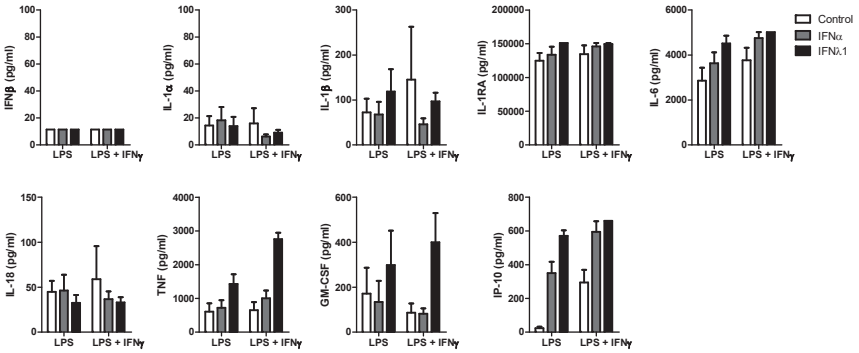
34 Tu, Z., Bozorgzadeh, A., Pierce, R. H., Kurtis, J., Crispe, I. N. and Orloff, M. S., TLR-dependent cross talk between human Kupffer cells and NK cells. J Exp Med 2008. 205: 233-244.

35 Biron, C. A., Nguyen, K. B., Pien, G. C., Cousens, L. P. and Salazar-Mather, T. P., Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol 1999. 17: 189-220.

36 Kakimi, K., Guidotti, L. G., Koezuka, Y. and Chisari, F. V., Natural killer T cell activation inhibits hepatitis B virus replication in vivo. J Exp Med 2000. 192: 921-930.

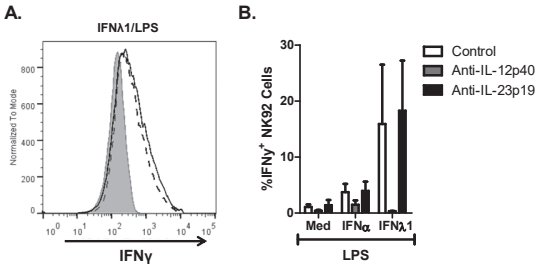
37 Kokordelis, P., Kramer, B., Korner, C., Boesecke, C., Voigt, E., Ingiliz, P., Glassner, A., Eisenhardt, M., Wolter, F., Kaczmarek, D., Nischalke, H. D., Rockstroh, J. K., Spengler, U. and Nattermann, J., An effective interferon-gamma-mediated inhibition of hepatitis C virus replication by natural killer cells is associated with spontaneous clearance of acute hepatitis C in human immunodeficiency virus-positive patients. Hepatology 2014. 59: 814-827.

38 Okamoto, M., Oshiumi, H., Azuma, M., Kato, N., Matsumoto, M. and Seya, T., IPS-1 Is Essential for Type III IFN Production by Hepatocytes and Dendritic Cells in Response to Hepatitis C Virus Infection. J Immunol 2014. 192: 2770-2777.



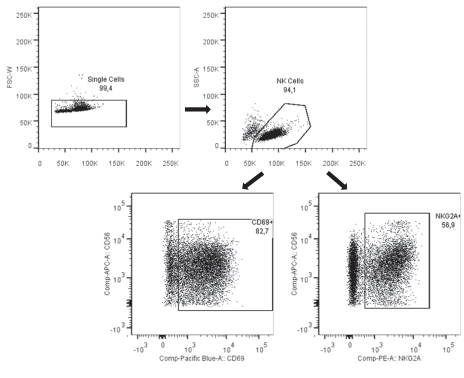
Supplementary Figure 1. Multiplex cytokine analysis of macrophage-associated cytokines and chemokines in IFN/TLR-stimulated macrophages

Multiplex analysis was performed on the supernatants of macrophages pre-incubated with IFNα or IFNλ1 for 5 hours and then further challenged using LPS, with (n = 8) or without (n = 13) IFNγ, for an additional 24 hours. Specific macrophage-associated cytokines and chemokines IFNβ, IL-1α, IL-1β, IL-1RA, IL-6, IL-18, GM-CSF, TNF, and IP-10 were selected as targets for analysis

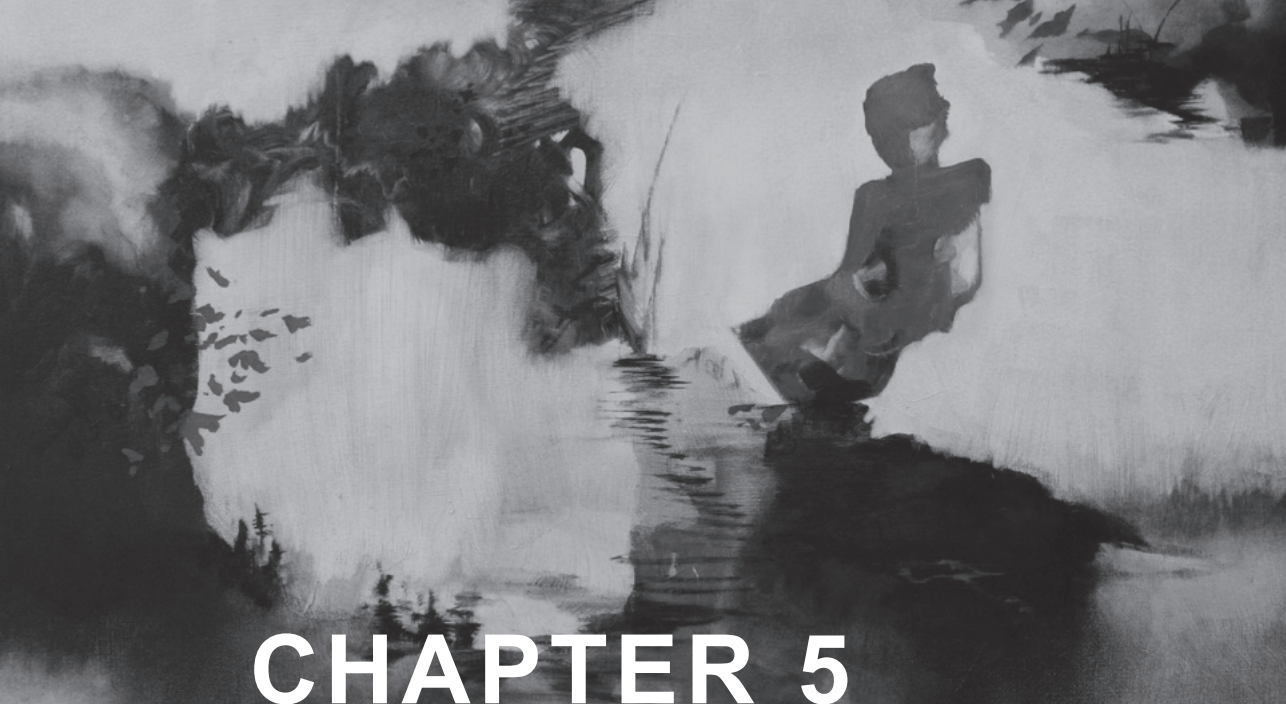


Supplementary Figure 2. IFNγ production by NK92 triggered by supernatant from IFNλ1/LPS-stimulated macrophages is IL-23 independent

NK92 cells were cultured with the supernatants of IFN and LPS stimulated macrophages in the presence of neutralizing antibodies against IL-12p40 and IL-23p19 (n = 3). All supernatants were supplemented with IL-18 during overnight stimulation, and intracellular IFNγ was measured using flow cytometry. (A) Representative histogram of macrophage-mediated IFNγ production by NK92 cells, cultured with supernatants of IFNλ1/LPS-stimulated macrophage (solid black line), in addition to IL-12p40 neutralization (filled light-grey line) or IL-23p19 neutralization (dashed black line). B) Collective results obtained from 3 independent donors.



Supplementary Figure 3. FACS gating strategy for CD69 and NKG2A expression on isolated human NK cells



CHAPTER 5

UNDERSTANDING IFN-λ IN RHEUMATOID ARTHRITIS

Rik A. de Groen[1], Bi-Sheng Liu[2], and André Boonstra[1]

[1] Department of Gastroenterology and Hepatology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[2] Department of Rheumatology, Leiden University Medical Center, Leiden, the Netherlands

ARTHRITIS RES THER. 2014 JAN 21;16(1):102.

ABSTRACT

Unraveling the mechanisms underlying the inflammatory response in rheumatoid arthritis is crucial in order to better understand the disease and to develop novel therapeutic approaches. Although the effect of type I IFNs on fibroblasts and in the context of rheumatoid arthritis has been described for some time, little is known on the effects of the type III interferons, also known as IFN-lambda. In this issue, Xu and collegues demonstrate that one of the members of the IFN-lambda family, IFNλ1 enhances TLR expression and consequently promotes the production of pro-inflammatory cytokines known to be involved in initiating and maintaining the inflammatory responses in rheumatoid arthritis.

Interferons (IFNs), known for their anti-viral, anti-proliferative, and immunomodulatory effects, are one of the immune system's first lines of defense against bacterial and viral infections. Three classes of IFNs have been described, designated type I, II, and III and are distinguished by their unique complimentary receptor complexes through which they signal (Figure 1). Type I IFNs, which are comprised of 13 IFNα subtypes as well as IFNβ, IFNω, and various others, engage through the IFNα receptor complex, composed of IFNαR1 and IFNαR2 chains. Type I IFNs have been well described and are used as a therapeutic for a myriad of diseases, including viral infections, auto-immune diseases, and even various forms of cancer. More recently, type III IFNs, also known as the IFNλs, have become of particular interest in the immunological field, with recent publications focusing on rheumatoid arthritis (RA) [1, 2]. IFNλ, comprised of IFNλ1, IFNλ2, IFNλ3, and IFNλ4, engage through the IFNλR1 and IL-10R2 complex. Despite triggering distinct receptor complexes, the downstream signaling of both type I and type III IFNs is regulated through JAK/STAT signal transduction, ultimately resulting in the induction of IFN-stimulated response elements (ISRE) and initiation of gene transcription. Opposed to the IFNα receptor, which is ubiquitously

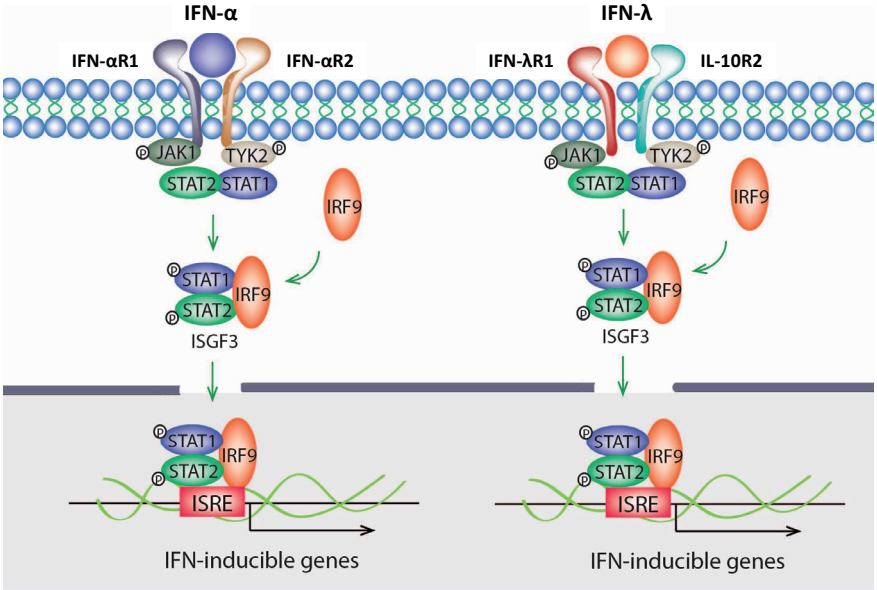


Figure 1: IFNα, and other type 1 IFNs, engage through the IFNα receptor complex, composed of IFNαR1 and IFNαR2, while the IFNλs signal through the IFNλR1 and IL-10R2 complex. Despite triggering distinct receptor complexes, the downstream signaling of both IFNα and IFNλ is regulated through JAK/STAT signal transduction, ultimately resulting in the induction of IFN-stimulated response elements (ISRE) and initiation of gene transcription. Opposed to the ubiquitously expressed IFNα receptor, the IFNλ receptor appears to be more limited in its expression.

expressed, the IFNλ receptor is more limitedly expressed potentially making it a more specific activator of immune responses.

When first described, it was suggested that IFNλ primarily acts on cells of epithelial origin [3], making it an activator of the innate immune response. Hepatocytes, also shown to be responsive to IFNλ stimulation [4], only became of particular interest after the discovery of single nucleotide polymorphisms (SNPs) located near the gene encoding for IFNλ3 that were associated with spontaneous as well as therapy-induced clearance of the hepatitis C virus [5], but also demonstrating that IFNλ's activity was not restricted to epithelial cells. The article by Xu et al. in this issue of Arthritis Research & Therapy describes the effects of IFNλ on fibroblasts and its context in RA [1]. Previously, the same research group demonstrated that IFNλ1 was expressed at higher levels in peripheral blood mononuclear cells (PBMC), serum, synovial fluid, and synovium in RA patients as compared to healthy individuals [2]. They now continue by showing that IFNλ1 is able to enhance TLR expression and consequently TLR-induced IL-6 and IL-8 production in the RA synovial fibroblasts (RA-FLS), contributing to RA synovial inflammation. Importantly, these effects are not only described in cell fibroblast cell lines, but also in primary fibroblasts (see supplementary figures). IFNλ and its modulation of TLR activation has also been described in monocyte-derived macrophages, where IFNλ incubation resulted in enhanced IL-12p40 and TNF production [6]. Outside of macrophages, B cells [7], and plasmacytoid dendritic cells [8] limited literature has described IFNλ and its effects on immune cells. Monocytes and natural kills cells, first reported to be IFNλ-responsive cellular subsets, have since been described as unresponsive [6, 9]. Due to an initial focus on anti-viral activity, IFNλ's immunological role and activity on immune cell populations still remains incomplete. However, increasing evidence suggests IFNλ also plays a larger role in immunoregulation.

Xu et al. convincingly show that IFNλ may have a detrimental effect for RA patients by enhancing to fibroblast-mediated pro-inflammatory cytokines, which may ultimately contribute to synovial inflammation. Similar effects have been described for IFNα in RA-FLS [10], and a large portion of literature has focused on the similarities between type I and III IFNs. The distinctions between these 2 classes of cytokines however remain almost completely undefined. Thus far, only differences between type I and III IFN have been reported in the regulation of pro-inflammatory cytokine production by macrophages [6], making it imperative to further investigate the unique qualities of IFNλ. The introduction of fibroblasts as an IFNλ-responsive population is an important finding, and may stimulate research on the underlying causes of inflammation in RA. Synovial-macrophages, another central population in RA research, have yet to be investigated for their response to IFNλ. This, in combination with the data presented by Xu et al. on RA-FLS, could give a more complete understanding of the immunological role of IFNλ in RA.

ACKNOWLEDGMENTS

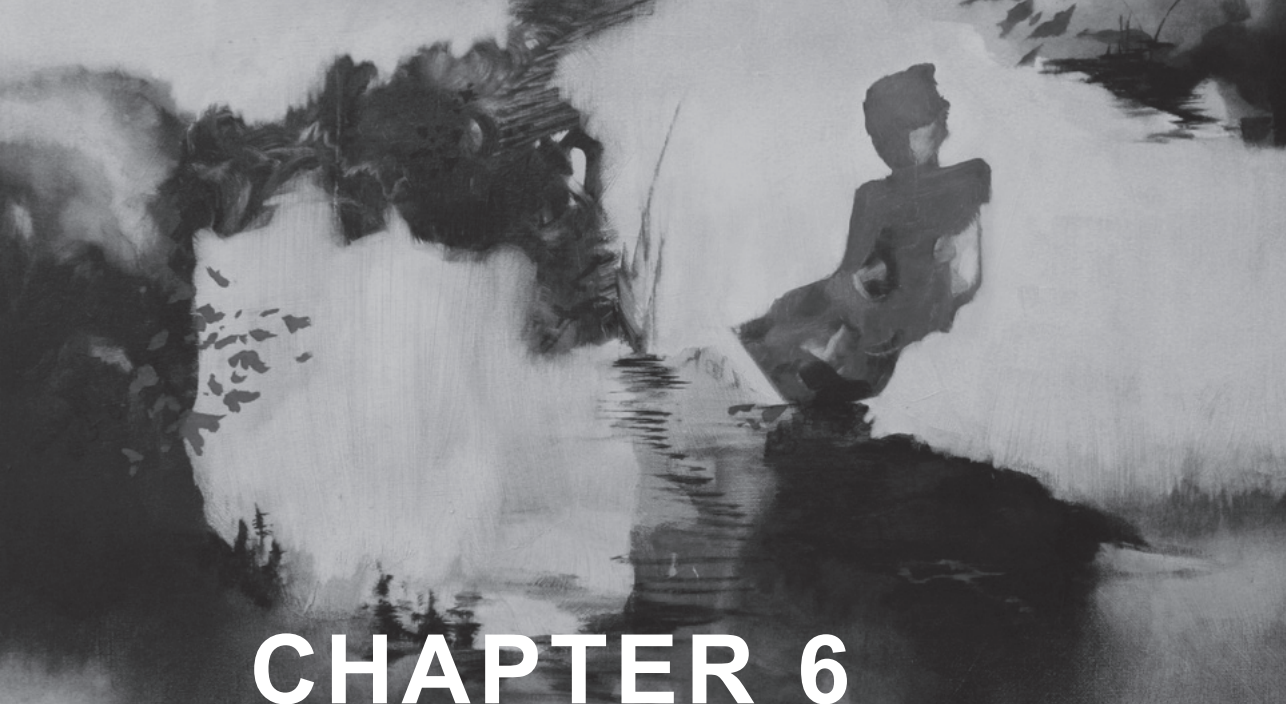
The studies by the authors supported by the Virgo consortium, funded by the Dutch government, project number FES0908, and by the Netherlands Genomics Initiative (NGI) project number 050–060–452.

REFERENCES

1. Xu LF, X.; Tan, W.; Gu, W.; Guo, D.; Zhang, M.; Wang, F.: IL-29 enhances Toll-like receptor-mediated IL-6 and IL-8 production by the synovial fibroblasts from rheumatoid arthritis patients. Arthritis Research & Therapy 2013.
2. Wang F, Xu L, Feng X, Guo D, Tan W, Zhang M: Interleukin-29 modulates proinflammatory cytokine production in synovial inflammation of rheumatoid arthritis. Arthritis Res Ther 2012, 14:R228.
3. Sommereyns C, Paul S, Staeheli P, Michiels T: IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. PLoS Pathog 2008, 4:e1000017.
4. Doyle SE, Schreckhise H, Khuu-Duong K, Henderson K, Rosler R, Storey H, Yao L, Liu H, Barahmand-pour F, Sivakumar P, et al: Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. Hepatology 2006, 44: 896-906.
5. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, et al: Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009, 461:399-401.
6. Liu BS, Janssen HL, Boonstra A: IL-29 and IFNalpha differ in their ability to modulate IL-12 production by TLR-activated human macrophages and exhibit differential regulation of the IFNgamma receptor expression. Blood 2011, 117:2385-2395.
7. Novak AJ, Grote DM, Ziesmer SC, Rajkumar V, Doyle SE, Ansell SM: A role for IFN-lambda1 in multiple myeloma B cell growth. Leukemia 2008, 22:2240-2246.
8. Megjugorac NJ, Gallagher GE, Gallagher G: Modulation of human plasmacytoid DC function by IFN-lambda1 (IL-29). J Leukoc Biol 2009, 86:1359-1363.
9. Kramer B, Eisenhardt M, Glassner A, Korner C, Sauerbruch T, Spengler U, Nattermann J: Do lambda-IFNs IL28A and IL28B act on human natural killer cells? Proc Natl Acad Sci U S A 2011, 108:E519-520; author reply E521-512.
10. Roelofs MF, Wenink MH, Brentano F, Abdollahi-Roodsaz S, Oppers-Walgreen B, Barrera P, van Riel PL, Joosten LA, Kyburz D, van den Berg WB, Radstake TR: Type I interferons might form the link between Toll-like receptor (TLR) 3/7 and TLR4-mediated synovial inflammation in rheumatoid arthritis (RA). Ann Rheum Dis 2009, 68:1486-1493.

PART 2

PHENOTYPE AND FUNCTIONAL RESPONSES OF NK CELLS IN VIRAL HEPATITIS INFECTION



CHAPTER 6

NK CELL PHENOTYPIC AND FUNCTIONAL SHIFTS COINCIDE WITH SPECIFIC CLINICAL PHASES IN THE NATURAL HISTORY OF CHRONIC HBV INFECTION

Rik A. de Groen[1,*], Jun Hou[1,*], Gertine W. van Oord[1], Zwier M.A. Groothuismink[1], Marieke van der Heide[1], Robert J. de Knegt[1], and André Boonstra[1]

[1] Department of Gastroenterology and Hepatology, Erasmus MC, University Medical Center Rotterdam, the Netherlands

* Both authors contributed equally

ANTIVIRAL RES. 2017 APR;140:18-24.

ABSTRACT:

Background: Chronic HBV infection can be divided into 4 distinct clinical phases: immune tolerant, immune active, inactive carrier, and HBeAg-negative hepatitis. Using a systems biology approach, we recently identified innate immune response components, specifically NK cells as a distinctive factor of specific HBV clinical phases. To expand on this study and identify the underlying immunological mechanisms, we performed a comprehensive profiling of NK cells in chronic HBV infection.

Methods: Peripheral blood from untreated chronic HBV patients was used to analyze phenotypic markers, as well as cytokine production and cytoxicity of NK cells.

Results: The overall composition, phenotype, and cytolytic activity of the NK cells remained constant across all clinical phases, with the exception of a few specific markers (KIRs, NKp46). $CD56^{bright}$ NK cells of chronic HBV patients differed in their ability to produce IFN-γ between the clinical phases pre- and post-HBeAg seroconversion.

Conclusion: This depicts a shift in NK cell characteristics between the immune active, under heavy viral or immune pressure, and inactive carrier phases, that coincides with HBeAg seroconversion. Although these changes in NK cells do not appear to be completely responsible for differences in liver damage characteristic of specific clinical phases, they could provide a step toward understanding immune dysregulation in chronic HBV infection.

INTRODUCTION

Infection with the hepatitis B virus (HBV) leads to non-cytopathic infections of the hosts' hepatocytes. Control of viral replication and subsequent liver injury are believed to be the consequence of the activity of the host immune response to infection. Contrary to infections with the hepatitis C virus (HCV), chronic HBV infections are characterized by episodes with differentiating serum levels of HBV DNA, alanine transferase (ALT), a marker of liver damage, and HBV envelope antigen (HBeAg). Using these parameters, different clinical phases have been discerned to describe the dynamics of the natural history of chronic HBV infection over a period of many years, and determine the indication for antiviral treatment on the basis of rate of viral replication and ALT elevation. Chronic HBV patients have been categorized into 4 clinical phases: the HBeAg-positive immune tolerant (IT) and immune active (IA) phases, as well as the HBeAg-negative inactive carrier (IC) and hepatitis (ENEG) phases (European Association For The Study Of The, 2012). The nomenclature to describe the natural course of HBV infection has led to confusion on the underlying mechanisms, as the IT phase accurately describes the situation where high levels of HBV DNA are observed without elevated ALT levels in serum, but erroneously suggests that the immune response is more tolerant to the presence of virus than in other phases. This, however, is not the case, since normal HBV-specific T cell responses are observed and no distinctive HBV-specific or global T cell activity could be identified in any of the clinical phases (Bertoletti and Hong, 2014; Park et al., 2016).

Over the last decade the importance of natural killer (NK) cells has been extensively described in chronic viral hepatitis (Rehermann, 2013). Various studies, including our own, have shown that chronic HBV patients and healthy individuals have similar total numbers of $CD56^{+}CD3^{-}$ NK cells in peripheral blood (Tjwa et al., 2011; Tjwa et al., 2014), whereas the ability of NK cells from patients to produce interferon (IFN)-γ is impaired (Boni et al., 2015; Lunemann et al., 2014; Oliviero et al., 2009; Peppa et al., 2010; Tjwa et al., 2011). However, a myriad of studies have reported conflicting results, observing no difference or even higher levels of IFN-γ production by NK cells in HBV patients compared to healthy individuals (Conroy et al., 2015; Li et al., 2014; Sun et al., 2012; Zhang et al., 2011). Part of the variation in outcome of these studies may be attributed to the large variety in stimuli (e.g. interleukin (IL)-2, IL-12, IL-15, IL-18, IFNα, PMA, and ionomycin) used alone or in combination to trigger IFN-γ production by NK cells. Also, the clinical and virological characteristics of the chronic HBV patients examined are likely to influence the features of the NK cell compartment, as many of the patient cohorts in these studies are an unsegregated mixture of all clinical phases (Lunemann et al., 2014; Oliviero et al., 2009; Peppa et al., 2010; Tjwa et al., 2011), whereas others examined either HBeAg-positive or HBeAg-negative patient groups (Boni et al., 2015; Conroy et al., 2015). This may be of particular

relevance, as *in vitro* studies have demonstrated that exposure of NK cells to HBeAg affected IL-18 receptor signaling, and consequently reduced the capacity to produce IFN-γ (Jegaskanda et al., 2014). In addition, HBV infection may alter the activation potential of NK cells by modulating the balance of activating and inhibitory receptors on the cell surface. During viral challenge the balance shifts from inhibition to activation after a critical threshold of activation signals exceeds those of inhibition (Lanier, 2005; Vivier et al., 2011). We previously showed that chronic HBV patients express elevated levels of the inhibitory receptor NKG2A and downregulated expression of activating receptors CD16 and NKp30 (Tjwa et al., 2011), although in general reports of the phenotype of NK cells differ between studies with vast degree of conflicting results (Mondelli et al., 2010).

We recently performed a systems biology study of peripheral blood transcriptomes in chronic HBV infection to better identify the mechanisms that govern the distinct clinical phases. Besides enhanced activity of IFN-stimulated genes (ISG) in the IT phase and B cell-function related genes in the IA phase, we also observed that upregulation of cytotoxicity/NK cell activity-related genes clustered in the IA and ENEG phase, i.e. the clinical phases with elevated ALT levels (Vanwolleghem et al., 2015). In the current study, we hypothesized that differential NK cell functionalities contribute to the distinct features observed during the HBV clinical phases, including the fluctuations in liver damage markers and HBV replication. Numerical, phenotypical, and functional analysis of immune parameters, of NK cells obtained from the 4 clinical phases was conducted to obtain an in depth profiling of NK cells throughout the course of natural history of chronic HBV infection.

MATERIALS AND METHODS

Patient selection and characteristics

Prospectively collected peripheral blood mononuclear cell (PBMC) samples from 40 31 untreated chronic HBV patients attending the outpatient hepatology clinic of the Erasmus MC (Rotterdam, The Netherlands) were selected, if there were no concomitant HIV, HCV, or HDV infections or oncological/rheumatological diseases. In addition, patients were excluded if they were pregnant, had significant steatosis on liver ultrasound, other liver pathology on liver biopsy, or had received antiviral treatment within the previous year. Liver fibrosis, as determined by histology or transient elastometry, was restricted to a maximum F2 Metavir score or maximum elasticity of 7.0 kPa. Based on serum HBV DNA, ALT levels, and HBeAg presence at the time of sampling, patients were categorized into 4 clinical HBV phases according to international guidelines (European Association For The Study Of The, 2012). Immune tolerant (IT) patients had detectable serum HBeAg and repetitive normal ALT values (< 40 U/L) for at least 1 year.

The HBeAg-positive immune active (IA) and HBeAg-negative (ENEG) patients had repetitive or intermittent abnormal serum ALT (> 40 U/L) values, and HBV DNA levels > 2,000 IU/mL. Inactive carrier (IC) patients were HBeAg-negative and had both repetitive normal ALT values (< 40 IU/L) and HBV DNA levels below 20,000 IU/ mL for at least 1 year. Serum ALT was measured on an automated analyzer, qualitative serum HBsAg and HBeAg levels were measured on an Architect Abbott analyzer, and serum HBV-DNA levels were measured using the COBAS AmpliPrep-COBAS Taq-Man HBVv2test (CAP-CTM; Roche Molecular Systems) (Chen et al., 2012; Feld et al., 2007; Papatheodoridis et al., 2012). Patient characteristics are presented in Table 1. PBMC were isolated from venous blood by ficoll separation (Ficoll-Paque^{TM} plus, Amersham), and stored at -150°C until used for the various assays. Written informed consent was obtained from all participants. The study protocol was approved by the institutional ethics committee and conducted in accordance with the guidelines of the Declaration of Helsinki.

Table 1: HBV patient characteristics

	Immune Tolerant (IT)	Immune Active (IA)	Inactive Carrier (IC)	HBeAg- Hepatitis (ENEG)
Sex (M/F)	3/4	4/2	5/3	9/1
Age (Years)	29.7 (24–47)	36.8 (18–89)	38.0 (30–08)	40.9 (29–97)
Ethnicity:				
Asian	7	5	5	4
African	0	0	3	3
Other	0	1	0	3
ALT	23.3 (14–49)	131.3 (64–429)	28.9 (8–89)	56.3 (29–93)
HBV DNA (IU/ml)	$5.7x10^8$ ($1.6x10^8$–$8.1x10^9$)	$3.8x10^8$ ($3.4x10^7$–$7.1x10^9$)	$1.5x10^3$ ($2.0x10^1$–$1.6x10^3$)	$1.3x10^6$ ($9.4x10^1$–$1.3x10^7$)
HBsAg (IU/ml)	52930 (150–03730)	36628 (6495–52570)	6152 (1–17970)	5439 (543–33466)
HBeAg	Positive	Positive	Negative	Negative
HBV Genotype:				
A	0	1	0	3
B/C	5	5	4	2
D	0	0	0	2
E	0	0	1	0
Unknown	2	0	3	3
Fibrosis:				
F0-F1	6	2	8	8
F1-F2	1	4	0	2

were measured using anti-CD3-PacificBlue (OKT3, eBioscience), anti-CD56-PE (MY31, BD), and anti-CD69-APC (L78, BD), followed by fixation with 2% formaldehyde, and permeabilization for intracellular staining with anti-IFN-γ-FITC (25723.11, BD). Activated and cytokine-producing NK cells were assessed by flow cytometry (FACS Canto II, BD) and analyzed using FlowJo version 10.1 (Tree Star Inc).

Statistical analysis

Data are expressed as the mean value ± SEM, unless indicated otherwise. The data were analyzed with Prism, version 5.0, software (GraphPad Software) using the Mann-Whitney U test to compare the variables between independent groups and the Spearman rank correlation coefficient test for nonparametric correlations. In all analyses, a 2-tailed $P < 0.05$ (95% confidence interval) was considered statistically significant.

RESULTS

Baseline characteristics of HBV patient cohort

In order to study differential immune compartments in HBV clinical phases, we carefully selected a cohort of untreated chronically HBV-infected patients, without any other co-morbidities, attending our outpatient clinic. To rule out the impact of advanced liver fibrosis on any identified immune parameters, patients with more than an F2 fibrosis score were excluded (Table 1). Typical for the natural history of chronic HBV infections, patients in the IT phase were the youngest group, followed chronologically by IA, IC, and ultimately ENEG patients. In addition, more females were represented in the asymptomatic IT and IA groups, given that they are more likely to be referred after routine HBsAg testing during pregnancy (Table 1). Although different ethnicities were observed in our tertiary hospital, most of the patients are of Asian ancestry. This is reflected by the fraction of patients infected with HBV genotypes B or C, excluding the ENEG group (Table 1). Owing to the stringent definition criteria, differences in ALT and HBV-DNA levels were observed between the clinical phases, and undetectable or unreactive HBeAg were measured in all IC and ENEG patients (Figure 1A). Furthermore, similar to previous reports (Jaroszewicz et al., 2010), quantitative HBsAg levels were lowest in IC patients and with a subtle increase seen again in patients in the ENEG phase (Figure 1B).

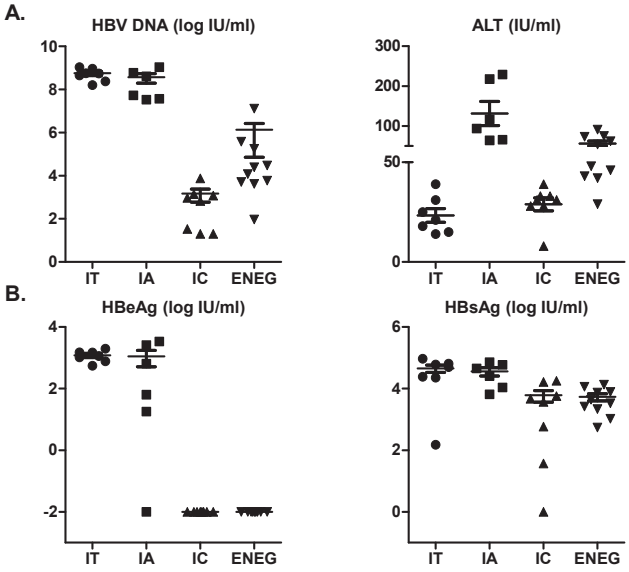


Figure 1. Baseline characteristics of chronic HBV patients separated into the four clinical phases based on HBV DNA, ALT, HBeAg, and HBsAg levels.
(A) Serum samples of 31 chronic HBV patients were assessed for their HBV DNA, ALT, (B) HBeAg, and HBsAg levels.

The frequencies of circulating NK cells and cytolytic molecule-expressing NK cells do not differ between the HBV clinical phases

To understand the role NK cells play in the natural course of chronic HBV infection, we assessed the phenotype and function of NK cells in the 4 specific clinical phases by flow cytometry. As shown in Figure 2A, we observed no significant differences in the percentage of total $CD56^{+}CD3^{-}$ NK cells, or the ratio of $CD56^{bright}$ and $CD56^{dim}$ subsets in the peripheral blood between any of the clinical groups.

Next, we performed intracellular analysis of the cytolytic mediators perforin, granzyme B, and TNF-related apoptosis-inducing ligand (TRAIL) (Figure 2B). No differences were observed in the expression of any of these cytotoxic markers in NK cells, with all expression levels remaining stable across the 4 clinical phases. In line with these findings, also K562-induced degranulation of NK cells did not differ (data not shown). Collectively, these results show no discerning factors in the total NK cell and NK cell subset frequencies, as well as expression of cytolytic mediators of NK cells in the different chronic HBV clinical phases.

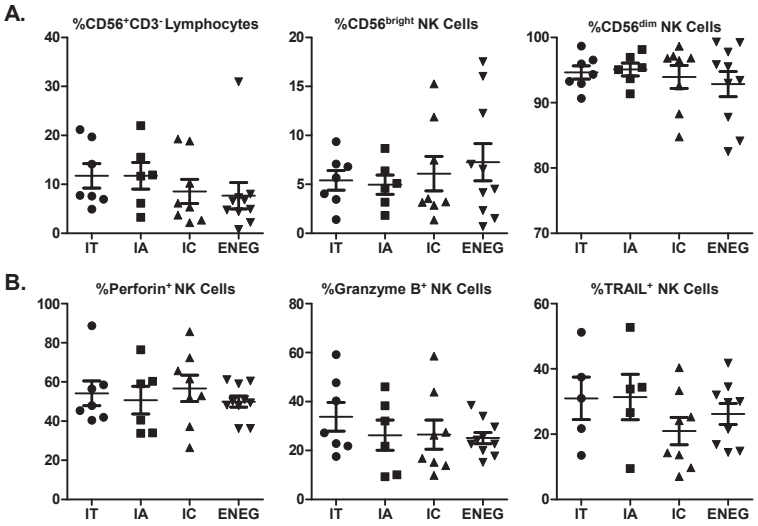


Figure 2. The frequency of circulating NK cells and cytolytic molecule-expressing NK cells do not differ between the clinical phases.

(A) Representative dot-plot showing the gating strategy for $CD56^{+}CD3^{-}$ NK cells in peripheral blood (B) The collective frequencies of $CD56^{+}CD3^{-}$ NK cells within the lymphocyte population of patients in the four different clinical phases, and the frequencies of $CD56^{bright}$ and $CD56^{dim}$ NK cells within the total NK cell population in PBMC of patients in the different clinical phases. (C) Intracellular perforin, intracellular granzyme B, and TRAIL expression were measured within the total NK cell compartment of the aforementioned groups. IT (n = 7), IA (n = 6), IC (n = 8), and ENEG (n = 10), all samples were evaluated by flow cytometry and data are shown as mean ± SEM. * denotes $p < 0.05$ and ** $p < 0.01$ (Mann-Whitney U test).

The frequencies of IFN-γ+/CD56bright expressing NK cells differ in IC patients when compared to the preceding clinical phases

To explore if the functionality of NK cells is correlated to a specific HBV clinical phase, we investigated the activation and cytokine-producing capacity of NK cells upon stimulation with IL-12 and IL-18 in the aforementioned chronic HBV patient cohort. As shown in Figure 3A and 3B, the percentages of CD69 and IFN-γ expressing total NK cells after stimulation were consistent across all 4 groups. However, when analyzing NK cell subsets a significant (1.3 fold) increase in the frequencies of IFN-γ-producing $CD56^{bright}$, but not $CD56^{dim}$ NK cells was observed in patients in the IC phases relative to those in the preceding IT and IA clinical phases (Figure 3C). Differential capacities for particular effector functions have long been described for the $CD56^{bright}$ and the $CD56^{dim}$ NK cell subsets, with $CD56^{bright}$ being able to produce higher levels of cytokine under certain conditions (Campbell et al., 2001; Cooper et al., 2001; Jacobs et al., 2001). This differential IFN-γ production observed in the $CD56^{bright}$ populations of IT and IA patients could therefore attribute to the distinct elevated viral load levels or expression of additional viral antigens (HBeAg) observed in these phases.

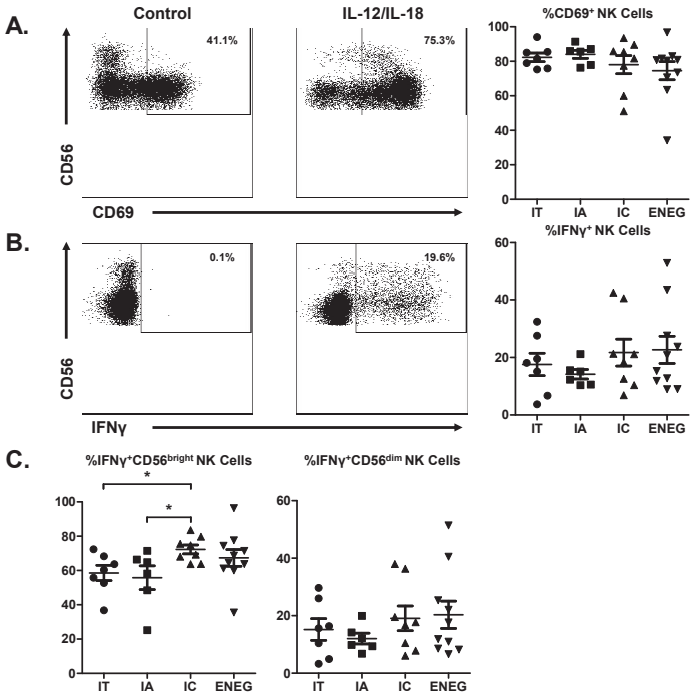


Figure 3. The frequency of IFN-γ^{+}/CD56^{bright} expressing NK cells differs in IC patients when compared to the preceding clinical phases.

(A) Representative dot-plots for the CD69 expression and (B) intracellular IFN-γ expression of NK cells, for PBMC either left unstimulated or in the presence of IL-12/IL-18 overnight, of chronic HBV patients in the IT, IA, IC and ENEG clinical phases. Collective results of multiple patients in each group shown in far right panels. (C) The frequency of IFN-γ-expressing NK cells was further divided into CD56^{bright} and CD56^{dim} NK subsets. IT (n = 7), IA (n = 6), IC (n = 8), and ENEG (n = 10), all samples were evaluated by flow cytometry and data are shown as mean ± SEM. * denotes $p < 0.05$ and ** $p < 0.01$ (Mann-Whitney U test).

NK cells of chronic HBV patients in the HBeAg-positive phases are characterized by reduced KIR and increased NKp46 expression as compared to the ENEG phase

NK cell activity is tightly regulated by a balance of activating and inhibitory receptors, and during viral infection the balance shifts from inhibition, the steady-state condition, toward activation (Lanier, 2005; Vivier et al., 2011). C-type lectin-like inhibitory receptor (CD94-NKG2A) and killer-cell immunoglobulin-like receptors (KIRs) comprise two of the main classes of inhibiting NK cell receptors, while NCR (e.g. NKp30, NKp46) and C-type lectin-like receptors (e.g. NKG2D) are two primary classes of activating NK receptors (Lanier, 2005). We therefore chose to investigate the expression of these receptors on NK cells in the setting of the natural history of HBV infection. Although the expression of activating NCR NKp30 remained stable across all 4 clinical phases, NK cells of ENEG patients

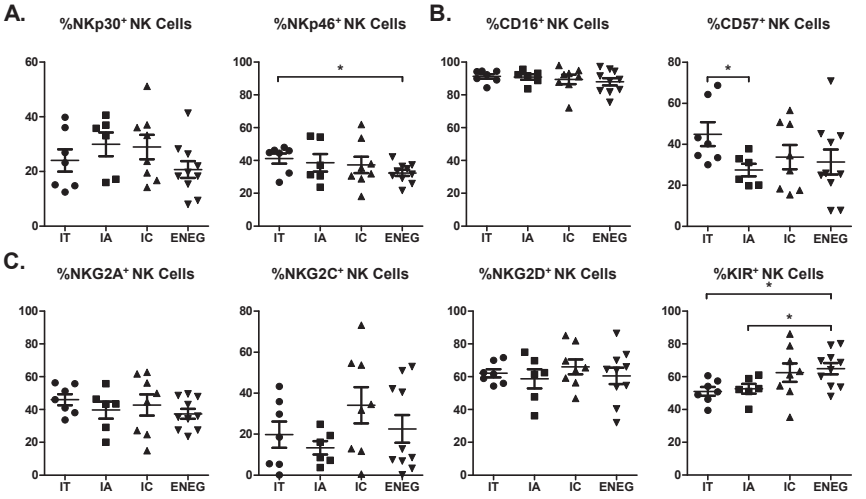


Figure 4. NK cells of chronic HBV patients in the HBeAg-positive phases are characterized by reduced KIR and increased NKp46 expression as compared to the ENEG phase.

Flow cytometric analysis was performed on the PBMC of chronic HBV patients in different clinical phases to determine the expression of (A) NCRs and activating receptors NKp30 and NKp46, (B) maturation-associated markers CD16 and CD57, (C) as well as a panel of KIRs and c-type lectin activating and inhibitory receptors NKG2A, NKG2C, and NKG2D on total NK cells. IT (n = 7), IA (n = 6), IC (n = 8), and ENEG (n = 10), all samples were evaluated by flow cytometry and data are shown as mean ± SEM. * denotes $p < 0.05$ and ** $p < 0.01$ (Mann-Whitney U test).

also express significantly lowers levels of activating NCR NKp46 than the IT group (Figure 4A). No differences were observed in the expression of inhibitory NKG2A, as well as activating NKG2D, C-type lectin receptors on NK cells between any of the IT, IA, IC, and ENEG phases (Figure 4C). The expression of KIRs, however, was significantly reduced (1.25 fold) on NK cells from patients in the HBeAg-positive IT and IA clinical phases relative to patients with ENEG hepatitis (Figure 4C).

Concurrently to investigating the activation and inhibitory potential of the NK cells in the varying HBV clinical phases, we chose to investigate the developmental phenotype of these cells using the specific differentiation-associated markers CD16, CD57, and NKG2C (Beziat et al., 2010; Bjorkstrom et al., 2010; Luetke-Eversloh et al., 2013). During analysis of these differentiation-associated receptors, only significant differences were observed when comparing CD57 in the two HBeAg-positive phases, with a significant reduction of CD57 expression seen in HBV patients in the IA clinical phase relative to the IT phase (Figure 4B). Additional analysis for all phenotypic markers was also performed on both the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets (Supplementary Figures 2–3). An increase in expression of activation receptor expression observed in $CD56^{bright}$ NK cells of IC patients, specifically NKp30 and NKG2D expression in respect to the earlier

HBeAg-postive IT and IA phases (IT only for NKG2D). Collectively these results suggest an increased inhibitory potential and reduced activation potential in the NK cells of ENEG patients compared to both HBeAg-positive phases, and a reduction of differentiated $CD57^{+}$ NK cells during the transition from the IT phase to the IA phase.

DISCUSSION

Previous modular transcriptome analysis of the whole blood of HBV patients identified NK cell/cytotoxicity activities as a distinctive marker in the elevated ALT phases, IA and ENEG, when compared to the IT phase (Vanwolleghem et al., 2015). To build on this study and to obtain a more detailed insight into the heterogeneity among chronic HBV patients, we examined, in the detail, NK cells throughout the course of the natural history of chronic HBV infection, specifically their potential causal role in the fluctuations of liver damage markers and HBV replication observed in these clinical phases. We demonstrate that (1) the overall composition, phenotype, and cytolytic activity of the NK peripheral cell compartment remains relatively constant across all clinical phases, with the exception of a few specific markers (e.g. KIRs, NKp46, and CD57), (2) $CD56^{bright}$ NK cells of chronic HBV patients differ in their ability to produce IFN-γ between the clinical phases pre- and post-HBeAg seroconversion.

The IA and ENEG clinical phases are characterized by elevated levels of ALT and liver damage. We hypothesized that this could partially be attributed to increased direct cytolytic activity and killing of infected hepatocytes by NK cells, but were surprised to find no measurable differences in the expression of cytotoxic mediators perforin, granzyme, and TRAIL across any clinical phases. In line with this, overall similarities were observed by flow cytometry in expression of NK cell activation (NKG2D, NKp30) and inhibitory receptors (NKG2A) when comparing the inflammatory (IA/ENEG) and non-inflammatory (IT/IC) phases. Furthermore, the lack of differences observed in the total percentage of NK cells in the lymphocyte compartment, as well as subset composition ($CD56^{bright/dim}$), suggests no increase in the proportion of NK cells or a phenotypic skewing toward the more cytolytic $CD56^{dim}$ subset. However, the significant changes observed in the proportion of NK cells expressing NKp46 and KIRs suggest an altered activation potential of NK cell compartments during specific clinical phases, markedly the ENEG phase. NK cells from patients in the terminal phase of the natural history of HBV infection, ENEG, expressed decreased levels of activating NCR NKp46 and enhanced levels of inhibitory, and markers of NK cell differentiation, KIRs, potentially resulting a decreased potential for activation for NK cells during this phase. Additionally, NKp46 has been shown to trigger NK cell activation and cytotoxicity upon ligation, but is expressed on mature NK cells irrespective of their activation in humans (Kruse et

al., 2014). Thus, the functional consequences of these observations are complex in nature, and could not be determined in the present study, since the characteristic NK cell parameters (granzyme, perforin, degranulation and TRAIL expression) were stable during the natural history of chronic HBV.

Although the NK cell compartment composition and expression of cytolytic mediators did not differ between clinical phases, one key distinction was the ability of $CD56^{bright}$ NK cells to produce IFN-γ upon cytokine stimulation. IFN-γ has been shown to be a trigger for the process of non-cytolytic HBV clearance and recruitment of inflammatory immune cells in both the innate and adaptive immune response to HBV infection (Bertoletti and Ferrari, 2016; Maini and Gehring, 2016). We demonstrate a differential capacity to produce IFN-γ in NK cells of chronic HBV patients pre- and post-HBeAg seroconversion, specifically between the IT/IA and IC phases. This differential production of IFN-γ observed could be the result of enhanced immune pressure due to high viral and antigen load present during the HBeAg-positive clinical phases, relieved after seroconversion and the subsequent viral suppression. However, we cannot say if this is a direct result of the high levels of viral particles and antigens present, or an indirect effect due to the coinciding immune activity in these phases.

Collectively, by conducting a detailed analysis of the phenotype and function of NK cells using clinically well-defined patient cohorts, we were able to characterize distinctive NK cell compartmental alterations during the progression of the chronic HBV natural history. Our data, obtained using peripheral blood, provide no evidence for higher NK cell activities during the IA and ENEG phase, thereby limiting the likelihood that NK cells are responsible for observed liver damage during these specific phases as reflected by fluctuating serum ALT levels. However, our findings show subtle changes indicative of a shift in NK cell phenotype and function between the IA phase under heavy viral or immune pressure, and the IC phase, that coincides with the process of HBeAg seroconversion. These observations shed light on the differences in the NK cell compartment during the natural history of chronic HBV, which may help better understand the extensive heterogeneity of immune responses observed in chronic HBV patients.

CONFLICT OF INTEREST

The authors of this manuscript have no conflicts of interest to declare.

ACKNOWLEDGMENTS

This work was supported by the Virgo consortium, funded by the Dutch government project number FES0908.

REFERENCES

Bertoletti, A., Ferrari, C., 2016. Adaptive immunity in HBV infection. J Hepatol 64, S71-83.

Bertoletti, A., Hong, M., 2014. Age-Dependent Immune Events during HBV Infection from Birth to Adulthood: An Alternative Interpretation. Front Immunol 5, 441.

Beziat, V., Descours, B., Parizot, C., Debre, P., Vieillard, V., 2010. NK cell terminal differentiation: correlated stepwise decrease of NKG2A and acquisition of KIRs. PLoS One 5, e11966.

Bjorkstrom, N.K., Riese, P., Heuts, F., Andersson, S., Fauriat, C., Ivarsson, M.A., Bjorklund, A.T., Flodstrom-Tullberg, M., Michaelsson, J., Rottenberg, M.E., Guzman, C.A., Ljunggren, H.G., Malmberg, K.J., 2010. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. Blood 116, 3853-3864.

Boni, C., Lampertico, P., Talamona, L., Giuberti, T., Invernizzi, F., Barili, V., Fisicaro, P., Rossi, M., Cavallo, M.C., Vecchi, A., Pedrazzi, G., Alfieri, A., Colombo, M., Missale, G., Ferrari, C., 2015. Natural killer cell phenotype modulation and natural killer/T-cell interplay in nucleos(t)ide analogue-treated hepatitis e antigen-negative patients with chronic hepatitis B. Hepatology 62, 1697-1709.

Campbell, J.J., Qin, S., Unutmaz, D., Soler, D., Murphy, K.E., Hodge, M.R., Wu, L., Butcher, E.C., 2001. Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. J Immunol 166, 6477-6482.

Chen, Y.C., Huang, S.F., Chu, C.M., Liaw, Y.F., 2012. Serial HBV DNA levels in patients with persistently normal transaminase over 10 years following spontaneous HBeAg seroconversion. J Viral Hepat 19, 138-146.

Conroy, M.J., Mac Nicholas, R., Grealy, R., Taylor, M., Otegbayo, J.A., O'Dea, S., Mulcahy, F., Ryan, T., Norris, S., Doherty, D.G., 2015. Circulating CD56dim natural killer cells and CD56+ T cells that produce interferon-gamma or interleukin-10 are expanded in asymptomatic, E antigen-negative patients with persistent hepatitis B virus infection. J Viral Hepat 22, 335-345.

Cooper, M.A., Fehniger, T.A., Turner, S.C., Chen, K.S., Ghaheri, B.A., Ghayur, T., Carson, W.E., Caligiuri, M.A., 2001. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. Blood 97, 3146-3151.

European Association For The Study Of The, L., 2012. EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. J Hepatol 57, 167-185.

Feld, J.J., Ayers, M., El-Ashry, D., Mazzulli, T., Tellier, R., Heathcote, E.J., 2007. Hepatitis B virus DNA prediction rules for hepatitis B e antigen-negative chronic hepatitis B. Hepatology 46, 1057-1070.

Jacobs, R., Hintzen, G., Kemper, A., Beul, K., Kempf, S., Behrens, G., Sykora, K.W., Schmidt, R.E., 2001. CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. Eur J Immunol 31, 3121-3127.

Jaroszewicz, J., Calle Serrano, B., Wursthorn, K., Deterding, K., Schlue, J., Raupach, R., Flisiak, R., Bock, C.T., Manns, M.P., Wedemeyer, H., Cornberg, M., 2010. Hepatitis B surface antigen (HBsAg) levels in the natural history of hepatitis B virus (HBV)-infection: a European perspective. J Hepatol 52, 514-522.

Jegaskanda, S., Ahn, S.H., Skinner, N., Thompson, A.J., Ngyuen, T., Holmes, J., De Rose, R., Navis, M., Winnall, W.R., Kramski, M., Bernardi, G., Bayliss, J., Colledge, D., Sozzi, V., Visvanathan, K., Locarnini, S.A., Kent, S.J., Revill, P.A., 2014. Downregulation of interleukin-18-mediated cell signaling and interferon gamma expression by the hepatitis B virus e antigen. J Virol 88, 10412-10420.

Kruse, P.H., Matta, J., Ugolini, S., Vivier, E., 2014. Natural cytotoxicity receptors and their ligands. Immunol Cell Biol 92, 221-229.

Lanier, L.L., 2005. NK cell recognition. Annu Rev Immunol 23, 225-274.

Li, Y., Wang, J.J., Gao, S., Liu, Q., Bai, J., Zhao, X.Q., Hao, Y.H., Ding, H.H., Zhu, F., Yang, D.L., Zhao, X.P., 2014. Decreased peripheral natural killer cells activity in the immune activated stage of chronic hepatitis B. PLoS One 9, e86927.

Luetke-Eversloh, M., Killig, M., Romagnani, C., 2013. Signatures of human NK cell development and terminal differentiation. Front Immunol 4, 499.

Lunemann, S., Malone, D.F., Hengst, J., Port, K., Grabowski, J., Deterding, K., Markova, A., Bremer, B., Schlaphoff, V., Cornberg, M., Manns, M.P., Sandberg, J.K., Ljunggren, H.G., Bjorkstrom, N.K., Wedemeyer, H., 2014. Compromised function of natural killer cells in acute and chronic viral hepatitis. J Infect Dis 209, 1362-1373.

Maini, M.K., Gehring, A.J., 2016. The role of innate immunity in the immunopathology and treatment of HBV infection. J Hepatol 64, S60-70.

Mondelli, M.U., Varchetta, S., Oliviero, B., 2010. Natural killer cells in viral hepatitis: facts and controversies. Eur J Clin Invest 40, 851-863.

Oliviero, B., Varchetta, S., Paudice, E., Michelone, G., Zaramella, M., Mavilio, D., De Filippi, F., Bruno, S., Mondelli, M.U., 2009. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. Gastroenterology 137, 1151-1160, 1160 e1151-1157.

Papatheodoridis, G.V., Manolakopoulos, S., Liaw, Y.F., Lok, A., 2012. Follow-up and indications for liver biopsy in HBeAg-negative chronic hepatitis B virus infection with persistently normal ALT: a systematic review. J Hepatol 57, 196-202.

Park, J.J., Wong, D.K., Wahed, A.S., Lee, W.M., Feld, J.J., Terrault, N., Khalili, M., Sterling, R.K., Kowdley, K.V., Bzowej, N., Lau, D.T., Kim, W.R., Smith, C., Carithers, R.L., Torrey, K.W., Keith, J.W., Levine, D.L., Traum, D., Ho, S., Valiga, M.E., Johnson, G.S., Doo, E., Lok, A.S., Chang, K.M., Hepatitis, B.R.N., 2016. Hepatitis B Virus-Specific and Global T-Cell Dysfunction in Chronic Hepatitis B. Gastroenterology 150, 684-695 e685.

Peppa, D., Micco, L., Javaid, A., Kennedy, P.T., Schurich, A., Dunn, C., Pallant, C., Ellis, G., Khanna, P., Dusheiko, G., Gilson, R.J., Maini, M.K., 2010. Blockade of immunosuppressive cytokines restores NK cell antiviral function in chronic hepatitis B virus infection. PLoS Pathog 6, e1001227.

Rehermann, B., 2013. Pathogenesis of chronic viral hepatitis: differential roles of T cells and NK cells. Nat Med 19, 859-868.

Sun, C., Fu, B., Gao, Y., Liao, X., Sun, R., Tian, Z., Wei, H., 2012. TGF-beta1 down-regulation of NKG2D/DAP10 and 2B4/SAP expression on human NK cells contributes to HBV persistence. PLoS Pathog 8, e1002594.

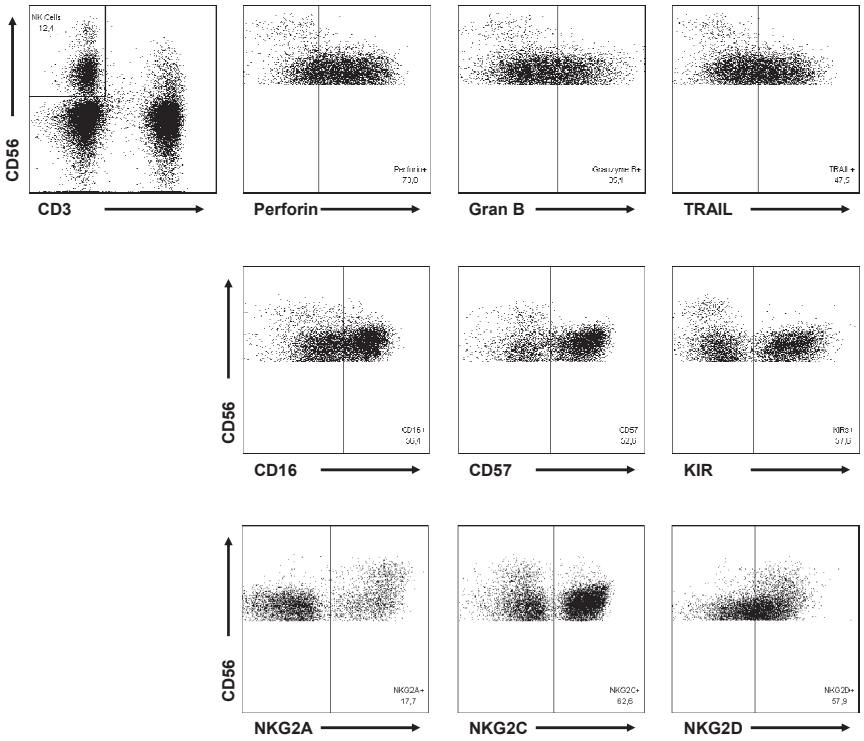
Tjwa, E.T., van Oord, G.W., Hegmans, J.P., Janssen, H.L., Woltman, A.M., 2011. Viral load reduction improves activation and function of natural killer cells in patients with chronic hepatitis B. J Hepatol 54, 209-218.

Tjwa, E.T., Zoutendijk, R., van Oord, G.W., Biesta, P.J., Verheij, J., Janssen, H.L., Woltman, A.M., Boonstra, A., 2014. Intrahepatic natural killer cell activation, but not function, is associated with HBsAg levels in patients with HBeAg-negative chronic hepatitis B. Liver Int 34, 396-404.

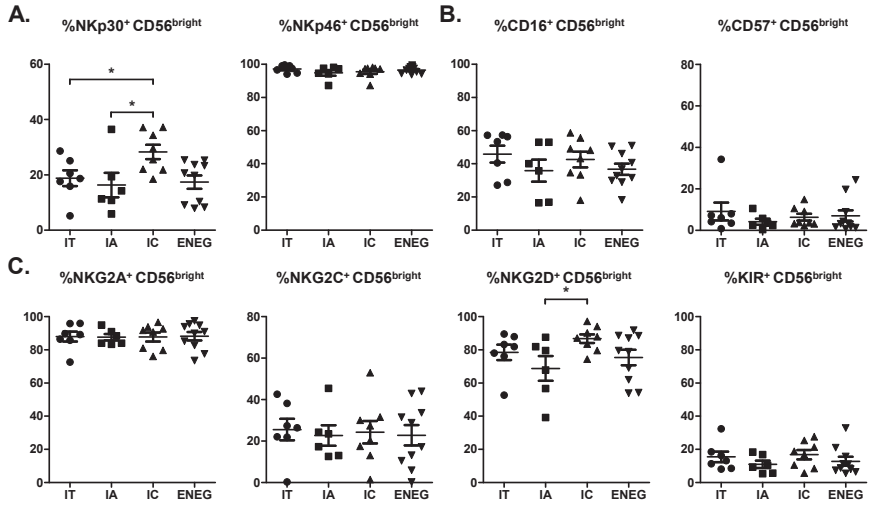
Vanwolleghem, T., Hou, J., van Oord, G., Andeweg, A.C., Osterhaus, A.D., Pas, S.D., Janssen, H.L., Boonstra, A., 2015. Re-evaluation of hepatitis B virus clinical phases by systems biology identifies unappreciated roles for the innate immune response and B cells. Hepatology 62, 87-100.

Vivier, E., Raulet, D.H., Moretta, A., Caligiuri, M.A., Zitvogel, L., Lanier, L.L., Yokoyama, W.M., Ugolini, S., 2011. Innate or adaptive immunity? The example of natural killer cells. Science 331, 44-49.

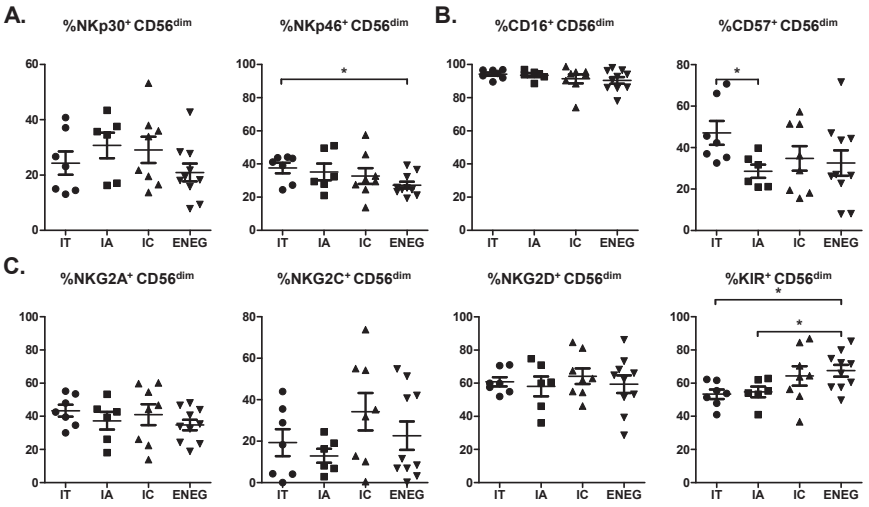
Zhang, Z., Zhang, S., Zou, Z., Shi, J., Zhao, J., Fan, R., Qin, E., Li, B., Li, Z., Xu, X., Fu, J., Zhang, J., Gao, B., Tian, Z., Wang, F.S., 2011. Hypercytolytic activity of hepatic natural killer cells correlates with liver injury in chronic hepatitis B patients. Hepatology 53, 73-85.



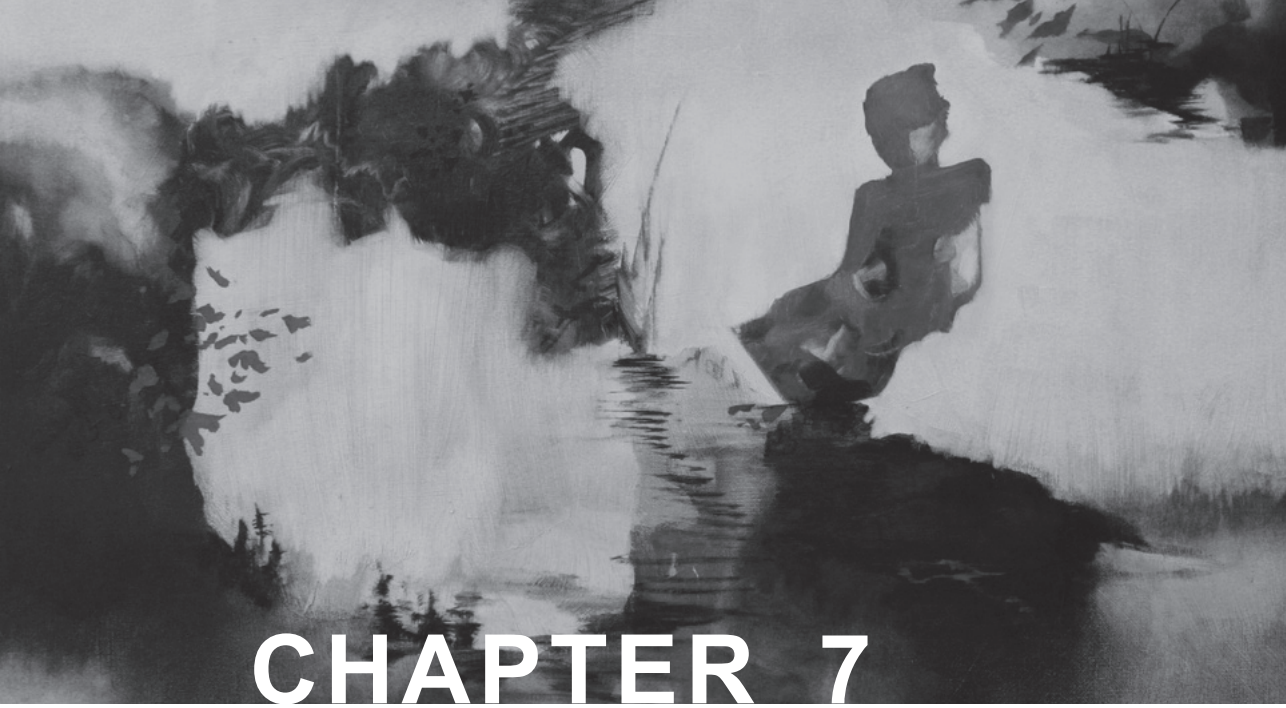
Supplementary Figure 1. Representative FACS plots for surface and intracellular molecules.



Supplementary Figure 2. Flow cytometric analysis was performed on the CD56bright NK cells in PBMC of chronic HBV patients in different clinical phases to determine the expression of (A) NCRs and activating receptors NKp30 and NKp46, (B) maturation-associated markers CD16 and CD57, (C) as well as a panel of KIRs and c-type lectin activating and inhibitory receptors NKG2A, NKG2C, and NKG2D on total NK cells. IT (n = 7), IA (n = 6), IC (n = 8), and ENEG (n = 10), all samples were evaluated by flow cytometry and data are shown as mean ± SEM. * denotes $p < 0.05$ and ** $p < 0.01$ (Mann-Whitney U test).



Supplementary Figure 3. Flow cytometric analysis was performed on the CD56bright NK cells inPBMC of chronic HBV patients in different clinical phases to determine the expression of (A) NCRs and activating receptors NKp30 and NKp46, (B) maturation-associated markers CD16 and CD57, (C) as well as a panel of KIRs and c-type lectin activating and inhibitory receptors NKG2A, NKG2C, and NKG2D on total NK cells. IT (n = 7), IA (n = 6), IC (n = 8), and ENEG (n = 10), all samples were evaluated by flow cytometry and data are shown as mean ± SEM. * denotes $p < 0.05$ and ** $p < 0.01$ (Mann-Whitney U test).



CHAPTER 7

NK CELLS IN SELF-LIMITED HCV INFECTION EXHIBIT A MORE EXTENSIVELY DIFFERENTIATED, BUT NOT MEMORY-LIKE, REPERTOIRE

Rik A. de Groen[1], Zwier M.A. Groothuismink[1], Gertine van Oord[1], Neeltje A. Kootstra[2,3], Harry L.A. Janssen[1,4], Maria Prins[2,3], Janke Schinkel[5], and André Boonstra[1]

[1] Department of Gastroenterology and Hepatology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[2] Public Health Service of Amsterdam, Department of Research, Cluster of Infectious Diseases, Amsterdam, the Netherlands
[3] Department of Internal Medicine, Division of Infectious Diseases, Amsterdam, the Netherlands
[4] Liver Clinic University Health Network, Division of Gastroenterology, University of Toronto, Canada
[5] Academic Medical Center, Department of Medical Microbiology, Section of Clinical Virology, Amsterdam, the Netherlands

J VIRAL HEPAT. 2017 APR 17. DOI: 10.1111/JVH.12716. [EPUB AHEAD OF PRINT]

ABSTRACT

Background: Natural killer (NK) cells have long been thought of as a purely innate immune cell population, but increasing reports have described developmental and functional qualities of NK cells that are commonly associated with cells of the adaptive immune system. Of these features, the ability of NK cells to acquire functional qualities associated with immunological memory and continuous differentiation resulting in the formation of specific NK cell repertoires has recently been highlighted in viral infection settings.

Methods/Results: By making use of a unique cohort of monitored, at risk intravenous drug users in this study, we were able to dissect the phenotypic and functional parameters associated with NK cell differentiation and NK cell memory in patients 3 years after acute HCV infection and either the subsequent self-clearance or progression to chronicity. We observed increased expression of cytolytic mediators and markers $CD56^{bright}$ and $NKp46^{+}$ of NK cells in patients with chronic, but not self-limited HCV infection. Patients with a self-limited infection expressed higher levels of differentiation-associated markers CD57 and KIRs, and lower levels of NKG2A.

Conclusions: A more extensively differentiated NK cell phenotype is associated with self-clearance in HCV patients, while the NK cells of chronic patients exhibited more naïve and effector NK cell phenotypic and functional characteristics. The identification of these distinct NK cell repertoires may shed light on the role NK cells play in determining the outcome of acute HCV infections, and the underlying immunological defects that lead to chronicity.

INTRODUCTION

Spontaneous clearance of an acute hepatitis C virus (HCV) infection occurs in approximately 25% of patients whom contract a primary infection (1). Clearance has been associated with strong T cell responses and a rapid induction of neutralizing antibodies against the autologous virus (2–5), but in recent years the importance of natural killer (NK) cells in this process has received renewed interest (6–9). NK cells act by eliminating HCV infection via two type of mechanisms; direct mechanisms, including the release of cytotoxic enzymes, such as granzymes and perforins, that result in lysis of the infected hepatocytes, and indirect mechanisms that involve the release of cytokines, such as interferon (IFN)-γ, by activated NK cells that result in triggering of antiviral responses that inhibit viral replication in infected cells.

NK cell activity is strictly governed by a balance of activating and inhibitory receptors on the cell surface. Some of the major classifications of these regulatory receptors include C-type lectin-like receptors (NKG2A, NKG2C, NKG2D), natural cytotoxicity receptors (NKp30, NKp44 and NKp46), and killer cell immunoglobulin-like receptor (KIRs) (10, 11). During viral infection the balance shifts from inhibition to activation after a critical threshold of activation signals exceeds those of inhibition (12). In addition to the regulation of the function of NK cells by these cell surface receptors, differentiation from $CD56^{bright}$ to $CD56^{dim}$ cells has also been shown to contribute to functional and phenotypic heterogeneity observed during the life span of NK cells. Many details of this differentiation process remain undefined, but the dynamics and outcome are likely affected by viral infections. During the differentiation process, NK cells lose expression of NKG2A, and subsequently acquire KIR and CD57 expression, ultimately resulting in a more terminally differentiated phenotype, that display reduced IFN-γ producing and proliferative capacities (13, 14).

Besides differentiation of NK cells, in recent years development of NK cells into memory-like cells has also been reported in both mice and humans, suggesting more similarities between NK cells and CD8+ T cells than originally thought (15, 16). In humans, a long-lived memory-like NK cell population was described at higher frequencies in CMV-seropositive individuals than CMV-seronegative individuals (17, 18). These cells were found to express high levels of the of C-type lectin NKG2C, in combination with maturation marker CD57 (17). A memory phenotype for NK cells has also been suggested in *Mycobacterium tuberculosis* and hantavirus (19, 20), but little information is available in HCV infections. This would, however, be highly relevant since in high-risk populations like injecting drug users (IDUs), reinfection is frequently observed in patients would have previously spontaneously cleared an HCV infection. The clinical observation that primary HCV infections are cleared in only a minority of individuals, while spontaneous clearance of an HCV reinfection occurs in approximately 80% of cases (21, 22), strongly suggests that immunity acquired during the primary infection

may play a role in the protection against HCV reinfection, and that some sort of immunological memory to HCV has developed.

With limited information available on the development and persistence of memory-like NK cells during and after HCV infection, we chose to characterize and compare the NK cell compartments of individuals who either resolved an acute HCV infection and individuals that developed a chronic infection. The aim of this study was to determine if memory-like NK cells are present following the resolution of a primary HCV infection, as well as whether the phenotypic and functional parameters associated with the NK cell differentiation process differ between the resolved patients and those chronically infected with HCV. By utilizing the unique cohort of self-clearing IDUs and the characterizing the effector and memory NK cell compartments in these individuals, we hope to provide immunological insight on how and to what extent NK cells might enhance immune-based antiviral strategies, such as vaccines and immunomodulators, in HCV infection.

MATERIALS AND METHODS

Patient selection and characteristics

Isolated peripheral blood mononuclear cells (PBMC) of 32 individuals were selected from archived samples collected in the Amsterdam Cohort Studies (ACS), which were in 1984 to investigate the epidemiological, behavioral, and psychosocial aspects of HIV/AIDS among homosexual men and drug users (23–25). In these studies participants were requested to visit the center every six months for HIV/HCV-screening, and were evaluated for at-risk behavior and various related psychosocial determinants. Blood was collected and stored for future studies. HCV RNA and antibody tests were performed retrospectively for all participants until 2003 and prospectively from October 2007 until 2010 for HIV-negative participants (26). The characteristics of the selected patients are presented in Table 1. All individuals selected were HIV negative/unexposed, and at-risk IDUs who were age and ethnically matched individuals who displayed similar risk behaviors, and who were reinfection-naïve. The individuals were categorized into 3 groups for cross-sectional analysis: 1) at-risk individuals that were never exposed to HCV infection and did have serological evidence of HCV infection at the time of sampling or at a later time-point (n = 10); 2) at-risk individuals that contracted a self-limiting acute HCV infection (viral clearance within first 6 months of infection) (n = 11); and 3) at-risk individuals that eventually developed a chronic infection (viral persistence 6 months post-infection) (n = 11). Using the virologic data provided by the bi-yearly visits of participants in the ACS, an approximate time of infection was determined for the cleared and chronic patients (± 6 months) and samples were selected approximately 3 years post-infection. Additionally, when available, samples were selected from the same self-clearing

Table 1. Participant characteristics at time of sample collection (approximately 3 years post-acute HCV infection*)

		Uninfected	Cleared HCV	Chronic HCV
Numbers		10	11	11
Sex	M/F	8/2	4/7	4/7
Age (yrs)		32.3 (25–59)	30.9 (24–42)	34.7 (24–47)
Ethnicity	Caucasian	10	8	9
	Asian	-	2	-
	Other	-	1	2
Time since infection (yrs)*		-	2.9 (2.4–4.0)	2.9 (2.0–0.8)
HCV bDNA (IU/ml)		-	< 615	$4.9x10^5$ ($6.3x10^3$–$3.9x10^6$)
Anti-HCV		negative	positive	positive
HCV genotype	genotype 1	-	1	6
	genotype 3		4	4
	genotype 4		1	1
	unknown		5	-

* estimated as time between first positive HCV bDNA test (with negative test at previous visit) and time of sample collection

and chronically infected individuals approximately 1 year post-infection for longitudinal analysis. The study was approved by the local ethics committee, and all patients and controls in the study gave informed consent before blood donation.

Expression of cell surface and intracellular molecules by flow cytometry

To determine the frequency and phenotype of peripheral blood NK cells, multi-color flow cytometry was performed on PBMC with anti-CD3-Alexa-Fluor700 (OKT-3, Beckman), anti-CD56-APC-eFluor780 (CMSSB, Beckman), anti-CD57-APC (HCD57, Biolegend), anti-KIR2D-Biotin (NKVFS1, Miltenyi biotech), anti-KIR3DL1/DL2-Biotin (5133, Miltenyi biotech), Streptavidin-eFluor450 (eBioscience), anti-NKG2A-PE (Z199, Beckman), anti-NKG2C-Alexa-488 (134591, R&D), anti-NKG2D-PerCP-Cy5.5 (1D11; BD), anti-NKp30-PE (Z25, Beckman), anti-NKp44-Biotin (P44–8, Biolegend), anti-NKp46-PE-Cy5 (BAB281, Beckman), anti-CD69-PacificBlue (FN50; Biolegend) and Live/Dead Aqua (Life Technologies). For intracellular expression of cytotoxic markers, PBMC ($0.5x10^6$ cells/200 µl) were fixed with 2% formaldehyde, and permeabilized for intracellular staining with anti-granzyme B-PE (GB11, eBioscience), anti-perforin-PerCP-eFluor710 (G9, eBioscience), and anti-TRAIL-Alexa488 (75402, R&D). Cells were again assessed by flow cytometry (FACS Canto II, BD) and analyzed using FlowJo version 10.1 (Tree Star Inc). Quadrants were set on negative expression of markers on $CD3^-CD56^-$ cells. Approximately 10^5 viable lymphocytes were collected for each individual sample.

Stimulation and intracellular cytokine analysis of NK cells

PBMC were stimulated with IL-12 (0.25 ng/ml) and IL-18 (10 ng/ml) overnight for surface marker expression and intracellular cytokine production. After 18 hours, brefeldin A (10 μg/ml, Sigma) was added to the cultures and the cells were incubated for an additional 3 hours. Cellular activation and surface markers were measured using anti-CD3-PacificBlue (OKT3, eBioscience), anti-CD56-PE (MY31, BD), and anti-CD69-APC (L78, BD), followed by fixation with 2% formaldehyde, and permeabilization for intracellular staining with anti-IFN-γ-FITC (25723.11, BD). Cytokine-producing cells were detected by flow cytometry (FACS Canto II, BD) and analyzed using FlowJo version 10.1 (Tree Star Inc). Quadrants were set on negative expression of CD69 on monocytes and the absence of IFN-γ production by B cells.

Statistical analysis

Data are expressed as the mean value ± SEM, unless indicated otherwise. The data were analyzed with Prism software, version 5.0 (GraphPad Software Inc) using the Mann-Whitney U test to compare the variables between independent groups and the Spearman rank correlation coefficient test for nonparametric correlations. In all analyses, a 2-tailed $p < 0.05$ (95% confidence interval) was considered statistically significant.

RESULTS

NK cells of patients with a chronic, but not self-limited, HCV infection have an increased $CD56^{bright}/CD56^{dim}$ ratio and expression of granzyme B and TRAIL

Previous studies have implicated a role for NK cells in the innate immune response to acute HCV infection (27, 28). To further dissect and understand the characteristics of NK cells following self-resolution of acute HCV infection or during progression to chronicity, we chose to perform a cross-sectional analysis of NK cell phenotype and function in uninfected individuals, patients with a self-limited HCV infection (cleared), and patients who developed a chronic infection. As a first step, using flow cytometric analysis, we evaluated the composition of PBMC, and showed that the percentage of total NK cells (defined by $CD56^{+}CD3^{-}$ expression) within the lymphocytes did not differ in the peripheral blood of any of the 3 groups (Figure 1A). However, an increase in the frequency of $CD56^{bright}$ and reduction of $CD56^{dim}$ expressing NK cells was observed in chronic patients as compared to both the uninfected and self-clearing individuals.

Concurrently with the compartmental analysis, the cytotoxic potential of NK cells from the uninfected, cleared, and chronic groups was assessed by intracellular analysis of the cytolytic mediators perforin, granzyme B, and TNF-related

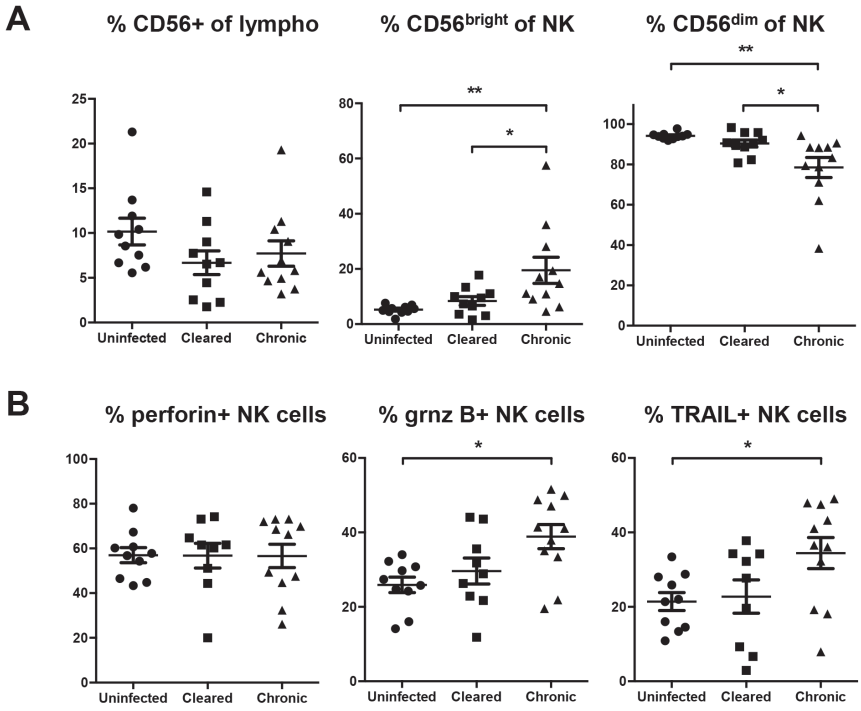


Figure 1. NK cells of patients with a chronic, but not self-limited, HCV infection have an increased $CD56^{bright}/CD56^{dim}$ ratio and expression of granzyme B and TRAIL

(A) Flow cytometric analysis was performed on the PBMC of uninfected, self-clearing, and chronic HCV patients to determine the frequency of total $CD56^{+}CD3^{-}$ NK cells in lymphocytes, and the frequency of $CD56^{bright}$ and $CD56^{dim}$ of these NK cells. (B) Intracellular flow cytometric analysis of perforin, granzyme B, and TRAIL was performed on the NK cells of the aforementioned patient groups. Data are shown as mean ± SEM. * denotes $p < 0.05$ and ** $p < 0.01$ (Mann-Whitney U test).

apoptosis-inducing ligand (TRAIL) (Figure 1B). In contrast to the frequency of perforin expressing NK cells, which remained stable across all groups, the frequencies of granzyme B and TRAIL expressing NK cells were significantly higher in chronic HCV patients compared to uninfected individuals or patients who had cleared the infection. Collectively, these results show an increase in $CD56^{bright}$ expression and cytolytic potential of NK cells from chronic HCV patients, but not self-limiting patients, when compared to uninfected individuals.

The frequency of cytokine induced IFN-γ production is unaltered in NK cells of self-limited and chronic HCV patients compared to uninfected individuals

Differential capacities for particular effector functions have been described for the $CD56^{bright}$ and the $CD56^{dim}$ NK cell subsets (29–31). To explore if the observed dif-

ferences in NK cell composition translated to differential NK cell effector function, we investigated the cytokine-producing capacity of these NK cells. PBMC from uninfected individuals, self-limited HCV patients, or chronic HCV were cultured in the presence of IL-12 and IL-18, and the surface expression of activation marker CD69, and intracellular expression of IFN-γ was determined (Figure 2A). The percentage of CD69 expressing NK cells after IL-12 plus IL-18 stimulation was consistent across all 3 groups (Figure 2A-B). Albeit not significant, a trend toward,

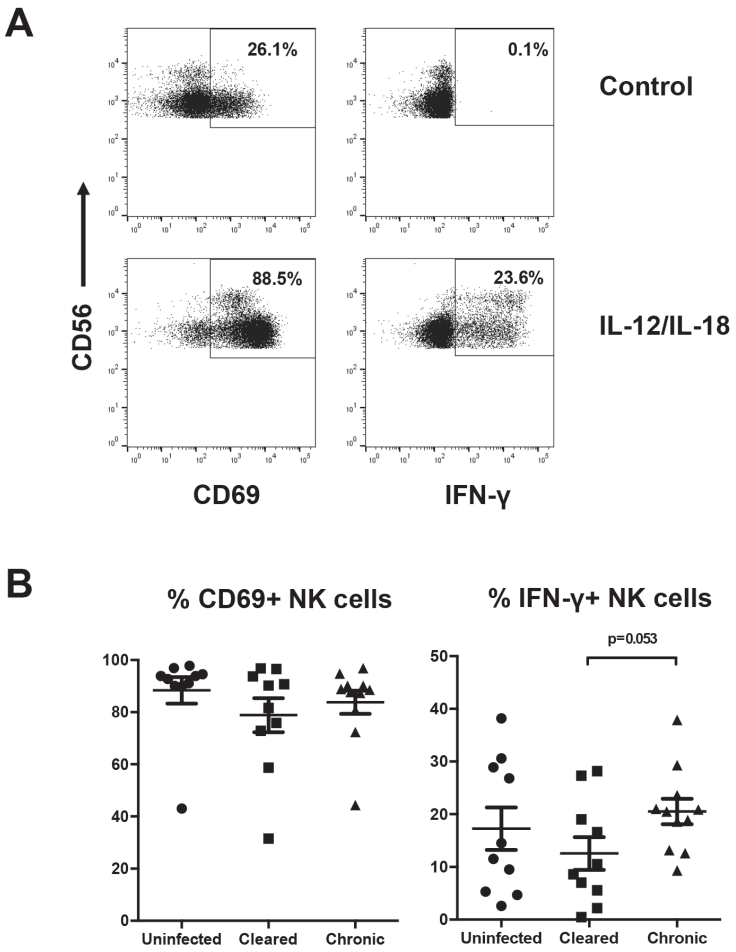


Figure 2. Frequency of IL-12/IL-18 induced IFN-γ production is unaltered in NK cells of self-limited and chronic HCV patients compared to uninfected individuals

(A) PBMC from uninfected, self-clearing, and chronic HCV patients were either left unstimulated (control) or stimulated with IL-12/IL-18 overnight, and surface/intracellular flow cytometric analysis was performed for the expression of CD69, and IFN-γ. (B) Collective results for multiple donors of CD69 and IFN-γ-positive cells after IL-12 and IL-18 stimulation. Data are shown as mean ± SEM. * denotes $p < 0.05$ (Mann-Whitney U test).

reduced frequencies of IFN-γ-producing NK cells was observed in patients with self-limited HCV relative to chronically infected patients. Neither the percentage of IFN-γ producing NK cells of self-clearing patients nor chronic HCV patients were significantly altered compared to uninfected individuals.

Expression of natural cytotoxicity receptors unaltered on NK cells of self-clearing HCV patients

NK cell activity is tightly regulated by a balance of activating and inhibitory receptors, and during viral infection, the balance shifts from inhibition, the steady-state condition, toward activation (10, 11). NCRs (eg. NKp30, NKp44, NKp46) and

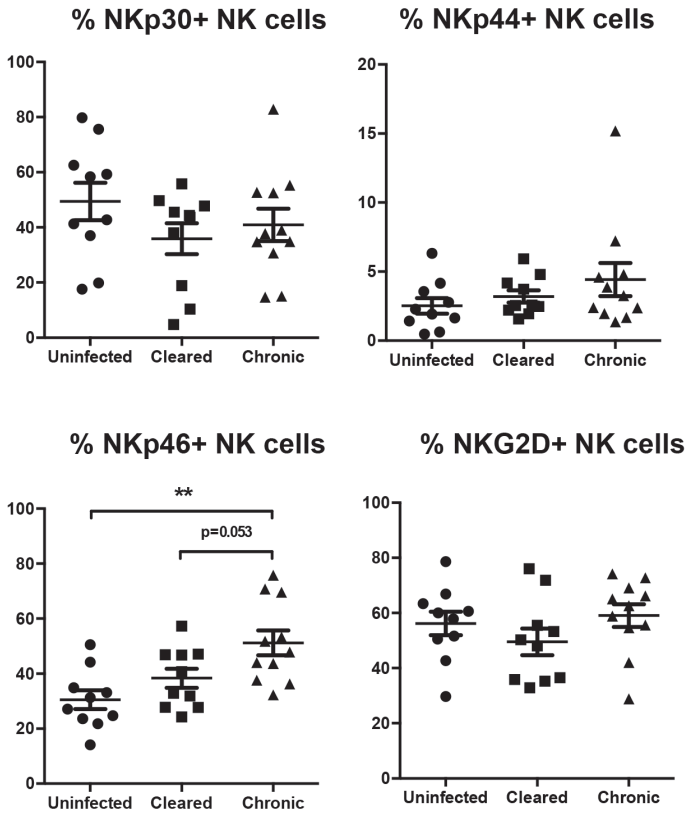


Figure 3. Expression of NKp30, NKp44, NKp46 and NKG2D is unaltered on NK cells of self-clearing HCV patients, whereas the frequency of NKp46^{+} NK cells is enhanced in patients with chronic HCV

Flow cytometric analysis was performed on the PBMC of uninfected, self-clearing, and chronic HCV patients to determine the expression of NKp30, NKp44, and NKp46, as well as NKG2D on total NK cells. Data are shown as mean ± SEM.* denotes $p < 0.05$ and ** $p < 0.01$ (Mann-Whitney U test).

C-type lectin-like receptors (eg. NKG2D) are two of main classes of activating NK receptors, and we therefore chose to investigate the expression of these receptors on NK cells in a self-limited and chronic HCV setting. Flow cytometric analysis showed no differential expression of NKp30, NKp44 or NKG2D on NK cells between the uninfected, cleared, and chronic groups (Figure 3). However, the frequency of NKp46-expressing NK cells was significantly higher in chronic HCV patients compared to uninfected controls, and showed a trend toward higher frequencies as compared to the NK cells of self-limited patients. Collectively, this data shows no difference in the expression of particular NCR and other activating receptors in self-clearing HCV patients compared to uninfected individuals, whereas NKp46 expression was elevated in chronic patients.

NK cell memory phenotype is not associated with self-clearance or chronic disease in HCV infection

The longstanding dogma of NK cells as a purely innate immune cell population has been challenged in recent years, with increasing reports describing qualities of NK cells that have commonly been associated with immunological memory in response to pathogens (15, 16). We therefore decided to investigate and characterize this unique NK cell population in HCV infection, and to determine its association with the resolution of acute HCV infections. First, the expression of maturation marker CD57 and the activating CD94-NKG2C receptor were assessed by flow-cytometry in uninfected individuals, as well as self-cleared and chronic HCV patients (Figure 4A). CD57 expression, but not NKG2C, was significantly greater on NK cells of self-limited HCV patients compared to uninfected controls, whereas in chronic patients CD57 and NKG2C expression remained unaltered.

Next, we examined the frequency of $CD57^{+}NKG2C^{++}$ expressing NK cells, as this population has been reported to represent the long-lived NK cell population in humans (17, 32, 33). Using flow cytometry, we were able to identify this population of NK cells, but did not observe a significant difference in the percentage of $CD57^{+}NKG2C^{++}$ expressing NK cells between uninfected individuals or either HCV patient group (Figure 4B). The expression of $CD57^{+}NKG2C^{++}$ NK cells remained stable from 1 to 3 years post-contraction of acute HCV infection in the cleared patients, and in chronic patients (Figure 4C), suggesting that this population is not diminished after viral clearance or during prolonged chronic infection. As a whole, these results show that despite an increased expression of CD57 on NK cells of self-limited HCV patients, this did not translate to differential frequencies of memory-associated NK cell populations in either self-cleared or chronic HCV patients.

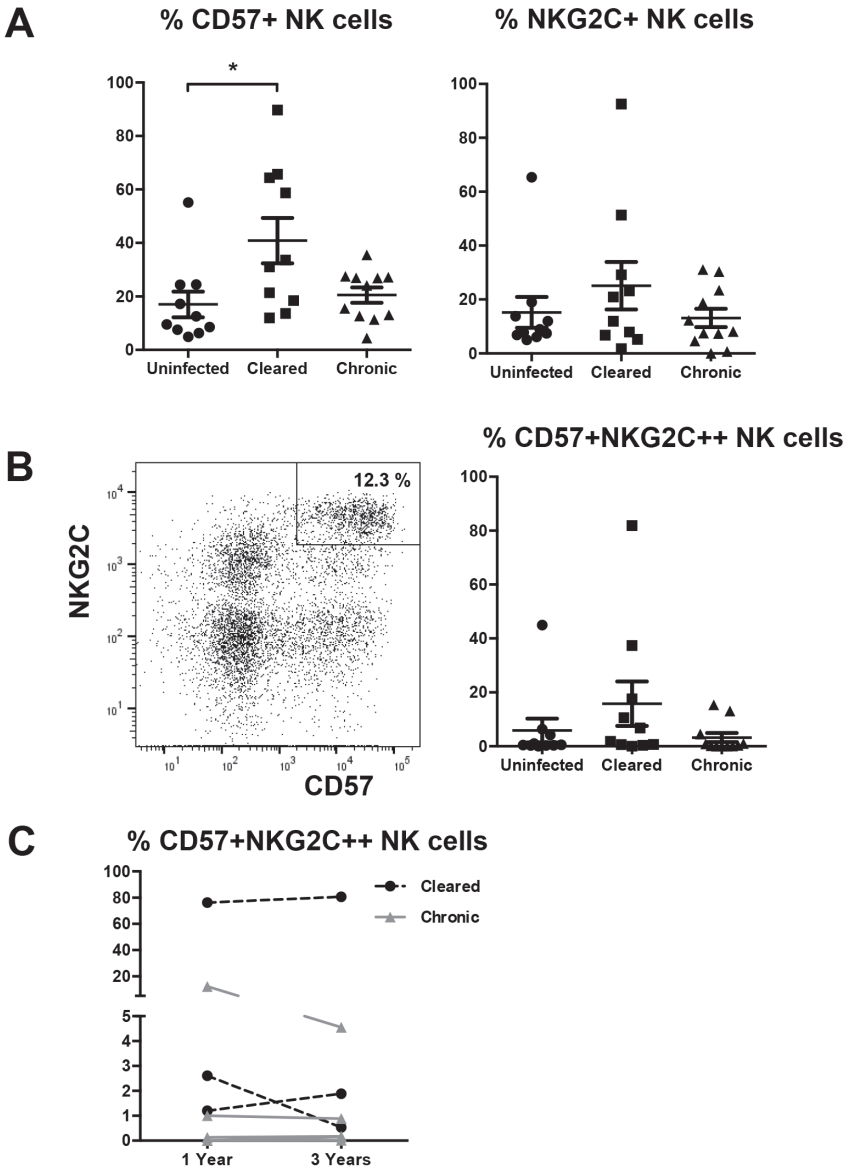


Figure 4. NK cell memory-associated phenotype is not associated with self-clearance or chronic disease in HCV infection

(A) NK cells in PBMC from uninfected, self-clearing, and chronic HCV patients were analyzed using flow cytometry for the expression of the NK cell maturation markers CD57 and NKG2C. (B) NK cells with a memory-associated phenotype were defined based on the expression of $CD57^{+}$ and $NKG2C^{++}$. Collective results for multiple donors of the frequency of these $CD57^{+}NKG2C^{++}$ NK cells. (C) Paired time points for patients at either 1 year or 3 years post infection. Data are shown as mean ± SEM. * denotes $p < 0.05$ and ** $p < 0.01$ (Mann-Whitney U test).

Self-limited HCV patient have more highly differentiated NK cell repertoires than uninfected and chronically infected individuals

The maturation marker CD57 expression has not only been linked to memory in NK cells, but it has also been implicated, in combination with inhibiting CD94-NKG2A receptor and KIRs, as defining markers in the differentiation of $CD56^{dim}$ NK cell repertoires (13, 14). To determine the differentiation status among NK cell populations and the role it may play in the clearance of HCV infection, we assessed the expression and co-expression of NKG2A, CD57 and KIRs. NK cells of self-limited HCV patients expressed significantly reduced levels of NKG2A compared to both uninfected and chronically infected individuals (Figure 5A). This reduction of NKG2A expression was only observed in $CD57^-$ NK cells, but not $CD57^+$ NK cells. KIR expression in the total NK cell compartment of cleared patients resembled that of uninfected individuals, whereas chronic patients expressed reduced levels of these receptors (Figure 5B). KIR expression in self-limited HCV patients appeared to be mainly attributed to the $CD57^+$ NK cells

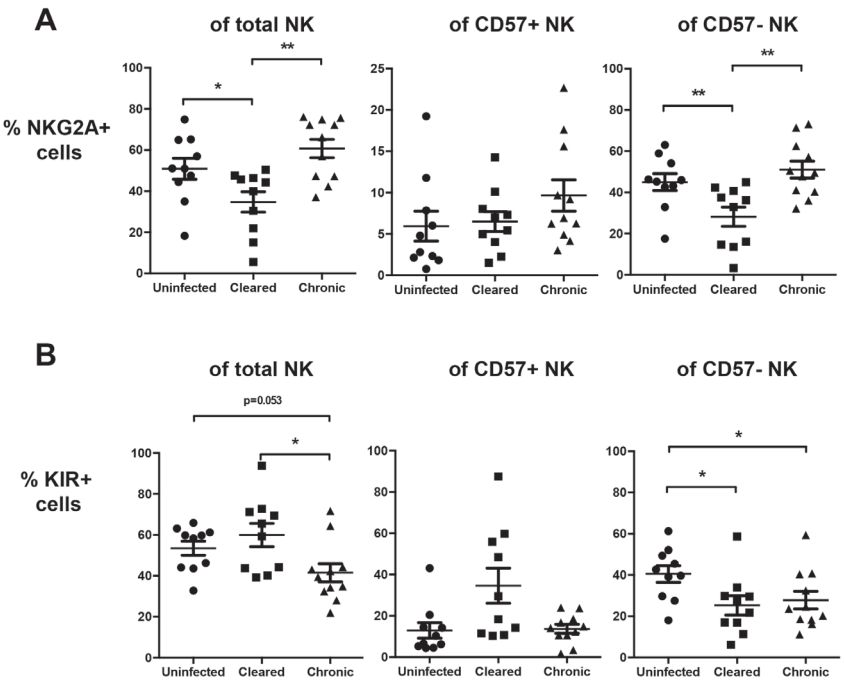


Figure 5. Self-limited HCV patients have more highly differentiated NK cell repertoires than uninfected and chronically infected individuals

NK cells in PBMC from uninfected, self-clearing, and chronic HCV patients were analyzed using flow cytometry for the expression of NKG2A and KIRs (KIR2D, KIR3DL1 and KIR3DL2). (A) NKG2A expression on total NK cells, and $CD57^+$ or $CD57^-$ NK cells (B) KIR expression on total NK cells, and $CD57^+$ or $CD57^-$ NK cells. Data are shown as mean ± SEM. * denotes $p < 0.05$ and ** $p < 0.01$ (Mann-Whitney U test).

and, similarly to the chronic patients, was significantly reduced on the $CD57^-$ NK population. NKG2A expression on NK cells was inversely correlated with both KIRs and CD57 expression only on the NK cells of the cleared group, whereas KIRs and CD57 were positively correlated in this subset of patients. Collectively this data suggests an enrichment of more highly differentiated $NKG2A^-CD57^+KIR^+$ cells in patients with a self-limiting HCV infection compared to uninfected and chronically infected individuals.

DISCUSSION

By making use of a unique cohort of monitored, at risk intravenous drug users in this study, we were able to dissect the phenotypic and functional parameters associated with NK cell differentiation and NK cell memory in patients 3 years after acute HCV infection and either the subsequent self-clearance of infection or progression to chronicity. Increased frequencies of NK cells expressing $CD56^{bright}$ and cytolytic mediators (granzyme B and TRAIL) were observed in chronically infected patients, but not those that cleared the acute infection, suggesting an overall more naïve and effector NK cell repertoire during chronic infection. NK cells repertoires of patients with a self-limited infection displayed a more highly differentiated phenotype, characterized by an increased CD57 and KIR expression, as well as reduced NKG2A expression, but did not exhibit a memory-like phenotype. Lastly, cytokine-induced activation, as seen by CD69, was comparable in all groups, whereas IFN-γ expressing NK cells showed a trend, but non-significant, decrease in self-limited patients as compared to the chronic group.

A key finding of this study was that, although, we were able to identify the memory-like NK cell population based on $CD57^+NKG2C^{++}$ expression in a subset of individuals from all groups in this study, no significant differences were observed in the total percentages of this specific NK cell repertoire. Additionally, the longitudinal analysis of both chronic and self-resolving patients from 1 to 3 years after estimated time of infection demonstrated a stable and unaltered expression and composition of this memory-like NK cell compartment. Our findings argue against the hypothesis that $CD57^+NKG2C^{++}$ NK cells observed after resolution of HCV infection are genuine memory NK cells induced by HCV exposure. Additionally, the protective features attributed to expanded expression of these populations upon re-exposure previously described in other viral infections, cannot justify the increased resistance to reinfection described in acute-resolved HCV patients.

Although we did not observe differences with respect to memory-like characteristics of NK cells, the phenotype and function of cells observed for acute-resolved patients did still drastically differ when compared to that of chronic patients and the uninfected group. Distinct alterations were observed between self-clearing patients and both the chronic and uninfected groups in respect to their expression

of maturation and differentiation-associated markers on NK cells. The expression of CD57 and KIRs were positively correlated (Supplementary Figure 1), and the expression of both these receptors was inversely correlated to that of NKG2A, suggesting a tight link between the regulation and expression of these receptors on the surface of NK cells of acute-resolved patients. This phenotypic alteration and regulation of markers resembles those previously described for NK cell differentiation [23, 24]. During this differentiation process, NK cells lose expression of NKG2A, and subsequently acquire KIRs and CD57, ultimately resulting in a more terminally differentiated phenotype that display reduced IFN-γ production and proliferative capacity. Our data indicates that NK cells of acute self-limited HCV patients persist in a more highly differentiated state, even years after the clearance of infection, although it is unclear if this is the cause or result of the resolution of the acute infection. However, the reduced proliferative and effector functionality described for terminally differentiated NK cells, makes it difficult to attribute the immunological resistance to reinfection in self-limited HCV patients to this NK cell repertoire.

In line with previous studies (34, 35), we observed an altered distribution of $CD56^{bright/dim}$ NK cell subsets in chronic HCV patients, but did not observe an overall decrease in the levels of NK cells as described by other studies (34, 36–38). The NK cells of these chronic patients also expressed significantly lower levels of CD16 (data not shown) and increased levels of NKp46 as compared to not only the uninfected group, but also the resolved patients. Increased NKp46 expression in this group is of particular interest, as it has been shown to be one of the principle avenues of NCR-mediated killing and correlated with the cytolytic activity of NK cells in HCV patients (39–41). This also supports skewing toward cytotoxicity, with increased frequencies of granzyme B and TRAIL expressing cells, observed in the chronic patients group. Interestingly, the frequencies of perforin expressing NK cells remained constant. This differential regulation of perforin versus granzyme B expressing NK cells has been described before by others, who showed distinct induction after triggering with IL-2, IL-4, PMA/ionomycin, and IFNα (42, 43).

Interestingly, however, some of these studies report depressed IFN-γ in chronic patients, whereas no differences were observed in the frequency of IFN-γ producing NK cells of chronic HCV patients in our study, but instead a trend toward reduction in the resolving HCV patients. This could be attributed to the highly differentiated phenotype of NK cells in this particular group, previously described to be impaired in their cytokine producing and proliferative capacities (13). Overall, our data, in combination with that previously described in literature, depicts NK cells in chronic patients as phenotypically naïve, with increased expression of NCRs and cytolytic mediators, reflecting the activation response to a persistent viral stimulation.

Collectively, our findings depict an altered, more extensively differentiated, but not memory-like, NK cell compartment in patients after resolving an acute infection, as compared to both uninfected individuals and patients with ongoing infections. This is in contrast to NK cells of chronic HCV patients that displayed a more naïve and effector phenotype with elevated cytotoxic capabilities. Although the highly differentiated NK cell repertoire in acute-resolved patients may not describe the resistance to reinfection observed in self-clearing acute HCV patients, the identification of these distinct NK cell repertoires may shed light on the role NK cells play in determining the outcome of secondary HCV infections, and the underlying immunological defects that lead to chronic infection.

FUNDING

This work was supported by the Virgo consortium, funded by the Dutch government project number FES0908.

ACKNOWLEDGMENTS

The Amsterdam Cohort Studies on HIV infection and AIDS, a collaboration between the Public Health Service of Amsterdam, the Academic Medical Center of the University of Amsterdam, Sanquin Blood Supply Foundation, Medical Center Jan van Goyen and the HIV Focus Center of the DC-Clinics, are part of the Netherlands HIV Monitoring Foundation and financially supported by the Center for Infectious Disease Control of the Netherlands National Institute for Public Health and the Environment (available at: http://www.amsterdamcohortstudies.org).

REFERENCES

1 Micallef JM, Kaldor JM, Dore GJ. Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. *J Viral Hepat*. 2006; 13(1): 34-41.

2 Claassen MA, Janssen HL, Boonstra A. Role of T cell immunity in hepatitis C virus infections. *Curr Opin Virol*. 2013; 3(4):461-7.

3 Claassen MA, Janssen HL, de Knegt RJ, Boonstra A. Controversy on the role of FoxP3(+) regulatory T cells in fibrogenesis in chronic hepatitis C virus infections. *J Hepatol*. 2014; 60(1):231-2.

4 Cooper S, Erickson AL, Adams EJ*, et al*. Analysis of a successful immune response against hepatitis C virus. *Immunity*. 1999; 10(4):439-49.

5 Pestka JM, Zeisel MB, Blaser E*, et al.* Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. *Proc Natl Acad Sci U S A*. 2007; 104(14):6025-30.

6 Gardiner CM. NK cell function and receptor diversity in the context of HCV infection. *Front Microbiol*. 2015; 6:1061.

7 Golden-Mason L, Rosen HR. Natural killer cells: multifaceted players with key roles in hepatitis C immunity. *Immunol Rev*. 2013; 255(1):68-81.

8 Lunemann S, Schlaphoff V, Cornberg M, Wedemeyer H. NK cells in hepatitis C: role in disease susceptibility and therapy. *Dig Dis*. 2012; 30 Suppl 1:48-54.

9 Spaan M, van Oord G, Kreefft K*, et al*. Immunological Analysis During Interferon-Free Therapy for Chronic Hepatitis C Virus Infection Reveals Modulation of the Natural Killer Cell Compartment. *J Infect Dis*. 2016; 213(2):216-23.

10 Vivier E, Raulet DH, Moretta A*, et al.* Innate or adaptive immunity? The example of natural killer cells. *Science*. 2011; 331(6013):44-9.

11 Lanier LL. NK cell recognition. *Annu Rev Immunol*. 2005; 23:225-74.

12 Mondelli MU, Varchetta S, Oliviero B. Natural killer cells in viral hepatitis: facts and controversies. *Eur J Clin Invest*. 2010; 40(9):851-63.

13 Beziat V, Descours B, Parizot C, Debre P, Vieillard V. NK cell terminal differentiation: correlated stepwise decrease of NKG2A and acquisition of KIRs. *PLoS One*. 2010; 5(8):e11966.

14 Bjorkstrom NK, Riese P, Heuts F*, et al.* Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood*. 2010; 116(19):3853-64.

15 O'Sullivan TE, Sun JC, Lanier LL. Natural Killer Cell Memory. *Immunity*. 2015; 43(4): 634-45.

16 Sun JC, Lopez-Verges S, Kim CC, DeRisi JL, Lanier LL. NK cells and immune "memory". *J Immunol*. 2011; 186(4):1891-7.

17 Lopez-Verges S, Milush JM, Schwartz BS*, et al.* Expansion of a unique CD57(+) NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proc Natl Acad Sci U S A*. 2011; 108(36):14725-32.

18 Beziat V, Dalgard O, Asselah T*, et al.* CMV drives clonal expansion of NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients. *Eur J Immunol*. 2012; 42(2):447-57.

19 Fu X, Yang B, Lao S, Fan Y, Wu C. Human memory-like NK cells migrating to tuberculous pleural fluid via IP-10/CXCR3 and SDF-1/CXCR4 axis produce IFN-gamma in response to Bacille Calmette Guerin. *Clin Immunol*. 2013; 148(1):113-23.

20 Bjorkstrom NK, Lindgren T, Stoltz M*, et al.* Rapid expansion and long-term persistence of elevated NK cell numbers in humans infected with hantavirus. *J Exp Med*. 2011; 208(1):13-21.

21 Osburn WO, Fisher BE, Dowd KA*, et al.* Spontaneous control of primary hepatitis C virus infection and immunity against persistent reinfection. *Gastroenterology*. 2010; 138(1):315-24.

22 Sacks-Davis R, Grebely J, Dore GJ*, et al.* Hepatitis C Virus Reinfection and Spontaneous Clearance of Reinfection—the InC3 Study. *J Infect Dis*. 2015; 212(9):1407-19.

23 Lambers FA, Prins M, Davidovich U, Stolte IG. High awareness of hepatitis C virus (HCV) but limited knowledge of HCV complications among HIV-positive and HIV-negative men who have sex with men. *AIDS Care*. 2014; 26(4):416-24.

24 Jansen IA, Geskus RB, Davidovich U*, et al.* Ongoing HIV-1 transmission among men who have sex with men in Amsterdam: a 25-year prospective cohort study. *AIDS*. 2011; 25(4):493-501.

25 van Griensven GJ, de Vroome EM, Goudsmit J, Coutinho RA. Changes in sexual behaviour and the fall in incidence of HIV infection among homosexual men. *BMJ*. 1989; 298(6668):218-21.

26 van de Laar TJ, van der Bij AK, Prins M*, et al.* Increase in HCV incidence among men who have sex with men in Amsterdam most likely caused by sexual transmission. *J Infect Dis*. 2007; 196(2):230-8.

27 Amadei B, Urbani S, Cazaly A*, et al.* Activation of natural killer cells during acute infection with hepatitis C virus. *Gastroenterology*. 2010; 138(4):1536-45.

28 Shoukry NH, Pelletier S, Chang KM. A view to natural killer cells in hepatitis C. *Gastroenterology*. 2011; 141(4):1144-8.

29 Campbell JJ, Qin S, Unutmaz D*, et al.* Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. *J Immunol*. 2001; 166(11):6477-82.

30 Cooper MA, Fehniger TA, Turner SC*, et al.* Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood*. 2001; 97(10):3146-51.

31 Jacobs R, Hintzen G, Kemper A*, et al.* CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. *Eur J Immunol*. 2001; 31(10):3121-7.

32 Foley B, Cooley S, Verneris MR*, et al.* Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand in vivo in response to recipient CMV antigen. *J Immunol*. 2012; 189(10):5082-8.

33 Lopez-Verges S, Milush JM, Pandey S*, et al.* CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood*. 2010; 116(19):3865-74.

34 Bonorino P, Ramzan M, Camous X*, et al.* Fine characterization of intrahepatic NK cells expressing natural killer receptors in chronic hepatitis B and C. *J Hepatol*. 2009; 51(3):458-67.

35 Golden-Mason L, Madrigal-Estebas L, McGrath E*, et al.* Altered natural killer cell subset distributions in resolved and persistent hepatitis C virus infection following single source exposure. *Gut*. 2008; 57(8):1121-8.

36 Oliviero B, Varchetta S, Paudice E*, et al*. Natural killer cell functional dichotomy in chronic hepatitis B and chronic hepatitis C virus infections. *Gastroenterology*. 2009; 137(3):1151-60, 60 e1-7.

37 Morishima C, Paschal DM, Wang CC*, et al*. Decreased NK cell frequency in chronic hepatitis C does not affect ex vivo cytolytic killing. *Hepatology*. 2006; 43(3):573-80.

38 Meier UC, Owen RE, Taylor E*, et al.* Shared alterations in NK cell frequency, phenotype, and function in chronic human immunodeficiency virus and hepatitis C virus infections. *J Virol*. 2005; 79(19):12365-74.

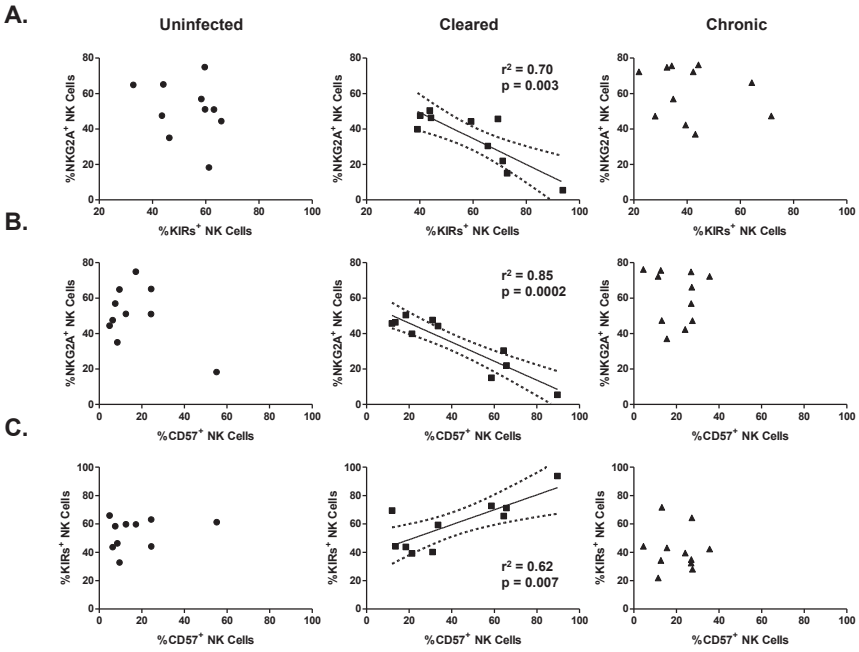
39 Mandelboim O, Lieberman N, Lev M*, et al*. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature*. 2001; 409(6823): 1055-60.

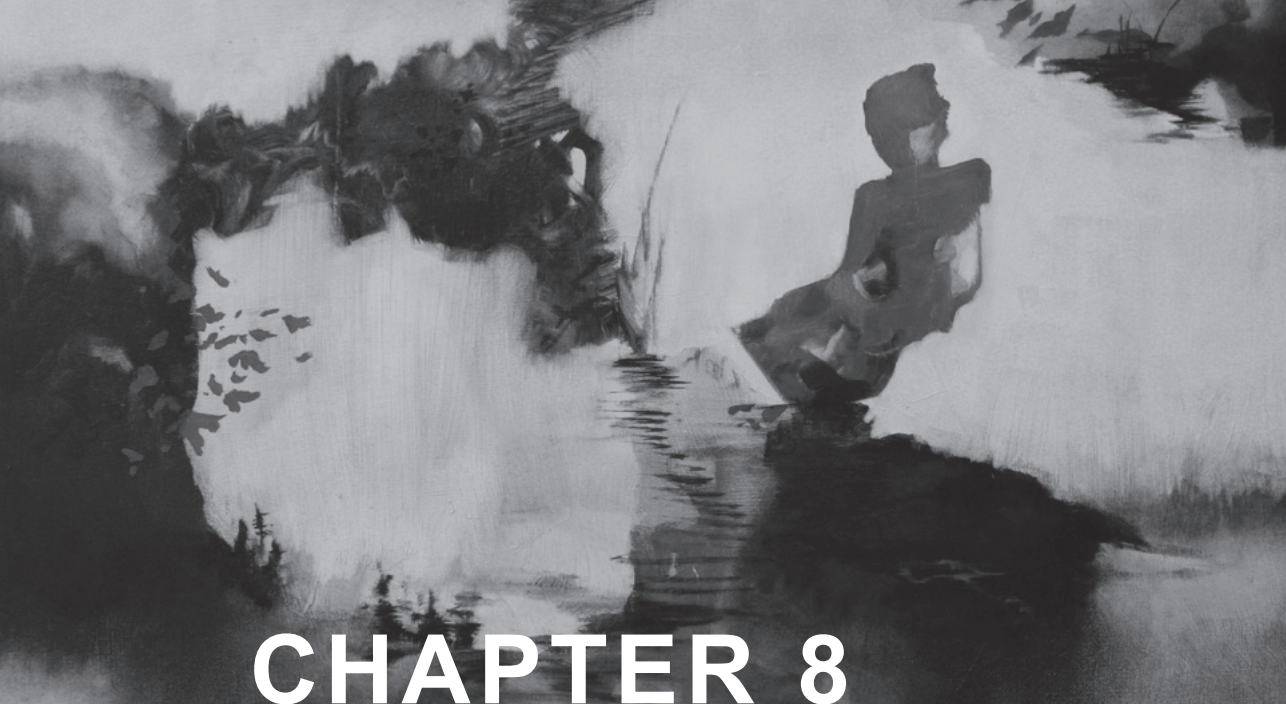
40 Sivori S, Pende D, Bottino C*, et al.* NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. *Eur J Immunol*. 1999; 29(5):1656-66.

41 Pembroke T, Christian A, Jones E*, et al.* The paradox of NKp46+ natural killer cells: drivers of severe hepatitis C virus-induced pathology but in-vivo resistance to interferon alpha treatment. *Gut*. 2014; 63(3):515-24.

42 Mori S, Jewett A, Cavalcanti M, *et al.* Differential regulation of human NK cell-associated gene expression following activation by IL-2, IFN-alpha and PMA/ionomycin. Int J Oncol. 1998; 12(5):1165-70.

43 Gardiner CM, Reen DJ. Differential cytokine regulation of natural killer cell-mediated necrotic and apoptotic cytotoxicity. Immunology. 1998; 93(4):511-7.


A.
B.
C.
Uninfected
Cleared
Chronic
%NKG2A+ NK Cells
%KIRs+ NK Cells
%CD57+ NK Cells
%KIRs+ NK Cells
r2 = 0.70
p = 0.003
r2 = 0.85
p = 0.0002
r2 = 0.62
p = 0.007



CHAPTER 8

SUMMARY AND DISCUSSION

HBV and HCV infection are the two leading causes of chronic liver inflammation worldwide, affecting an approximate 370–390 million people.[1–3] Currently, the viral and immunological mechanisms that underlie either the resolution or persistence of viral hepatitis infections are not fully understood. Investigation and identification of the integral components in the host immune responses that cumulatively lead to the effective clearance of the virus or development of a chronic infection are of the utmost importance. Therefore, the overall aim of this thesis was to understand the function, and dysfunction, of specific aspects of the innate immune system in the resolution or persistence of viral hepatitis infection. The two main components of the immune response focused on in this thesis were the roles IFNs, specifically type I and III IFNs, and NK cells play in determining disease state and outcome.

SYSTEMIC LEVELS AND IMMUNOLOGICAL REGULATION OF TYPE III IFNS IN VIRAL HEPATITIS INFECTION

Endogenous IFN-λ in viral hepatitis patients

Endogenous IFNs, specifically IFN-α, play a pivotal role in the innate immune response to viral infections and for many years represented a cornerstone in the treatment of chronic HCV and HBV infection until the recent introduction of DAAs. Type III IFNs, however, have only received increased attention since the discovery of SNPs within the IFN-λ gene locus that are associated with spontaneous as well as IFN-α/ribavirin therapy-induced clearance of HCV infection.[4–7] More recent publications have attempted to explain these associations, specifically with the identification of IFN-λ4 and its association with HCV resolution,[8] but the exact mechanism still remains unclear. Therefore, in Chapter 2 we performed a detailed analysis of the endogenous levels of type III IFNs in viral hepatitis patients and how these related to patient IFN-λ-polymorphism genotypes as well as disease state. We demonstrate that serum IFN-λ levels are not affected by infection with either HCV or HBV, and that IFN-λ levels are not associated with specific disease stages, outcome, or other parameters (e.g. ALT and IFN-λ3 rs12979860 genotype). However, this does not exclude endogenous IFN-λ expression from being, at least partially, responsible for the associations seen between SNPs within the IFN-λ gene locus and viral clearance. In this study, we investigated the systemic levels of IFN-λ by using serum from viral hepatitis patients, but this does not always reflect levels within specific tissues (e.g. intrahepatic levels). Studies have shown that IFN-λ3 is one of the predominant IFNs produced by hepatocytes during acute HCV infection.[9] Differences in the systemic levels of IFN-λ may not fully reflect the production of IFN-λ by hepatocytes at the site of infection, but instead the production by cells in the peripheral compartment, such

as pDCs and BDCA3$^+$ mDCs, known to be producers of IFN-λ during HCV infection.[10,11] These DC populations have been shown to be potent producers of type III IFNs, but still represent a relatively small fraction of IFN-producing cells when compared to hepatocytes. Additionally, a series of studies have reported impaired type I IFN, but not type III IFN, signaling in hepatocytes during HCV infection,[12,13] stressing the importance of not only the absolute levels, but also the differences in sensitivity of response by hepatocytes and other cell types to IFNs. These results, in combination with the differential gene transcript expression, kinetics, as well as cytokine production that have been described for IFN-α and IFN-λ in/by hepatocytes and other tissue resident cells (e.g. macrophages in Chapter 4), further stress the potential importance of intrahepatic IFN-λ levels during HCV infection.[14–17] Collectively, differences in intrahepatic IFN-λ expression and the sensitivity of hepatocytes, as well as other liver-resident cells, to this class of IFNs could at least partially explain the associations between SNPs within the IFN-λ gene locus and viral clearance in HCV patients. How this compares to and why this may differ in other viral infection settings, such as chronic HBV, is still not known. Although some of the same SNPs within the IFN-λ gene locus have been associated with IFN-α treatment response in HBeAg-positive chronic HBV patients,[18,19] these links appears to be much less pronounced. This could have to do with differential triggering of immune responses by the two hepatitis viruses, partially reflected in the strong ISG response characteristic of HCV infection, but almost completely devoid in chronic HBV infection. Additionally, cure rates of IFN-based therapies are significantly lower in chronic HBV as compared to HCV patients, suggesting a higher degree of susceptibility of HCV to the antiviral and immunomodulatory effects of IFNs.

Immunological regulation of type III IFNs

In addition to the implications of endogenously produced IFN-λ in viral hepatitis patients, much of the underlying basic biology of type III IFNs remains unexplained. The antiviral aspects of this class of cytokines have been well established, with many similarities described to type I IFNs. However, as previously stated, this then raises the question; what is the evolutionary benefit of having two classes of IFNs if their immunological roles are truly redundant? Therefore, further investigation is needed to understand the distinctions described for type III IFNs, specifically in regards to the IFN-λ receptor and its expression on immune cells as well as the associated responses after triggering. The collective aim of Chapters 3, 4, and 5 was to further explore the immunological regulation of IFN-λ on specific aspects of the innate immune response to viral infections (e.g. pathogen-mediated B cell, macrophages, and NK cells responses), and how this compares to that of IFN-α.

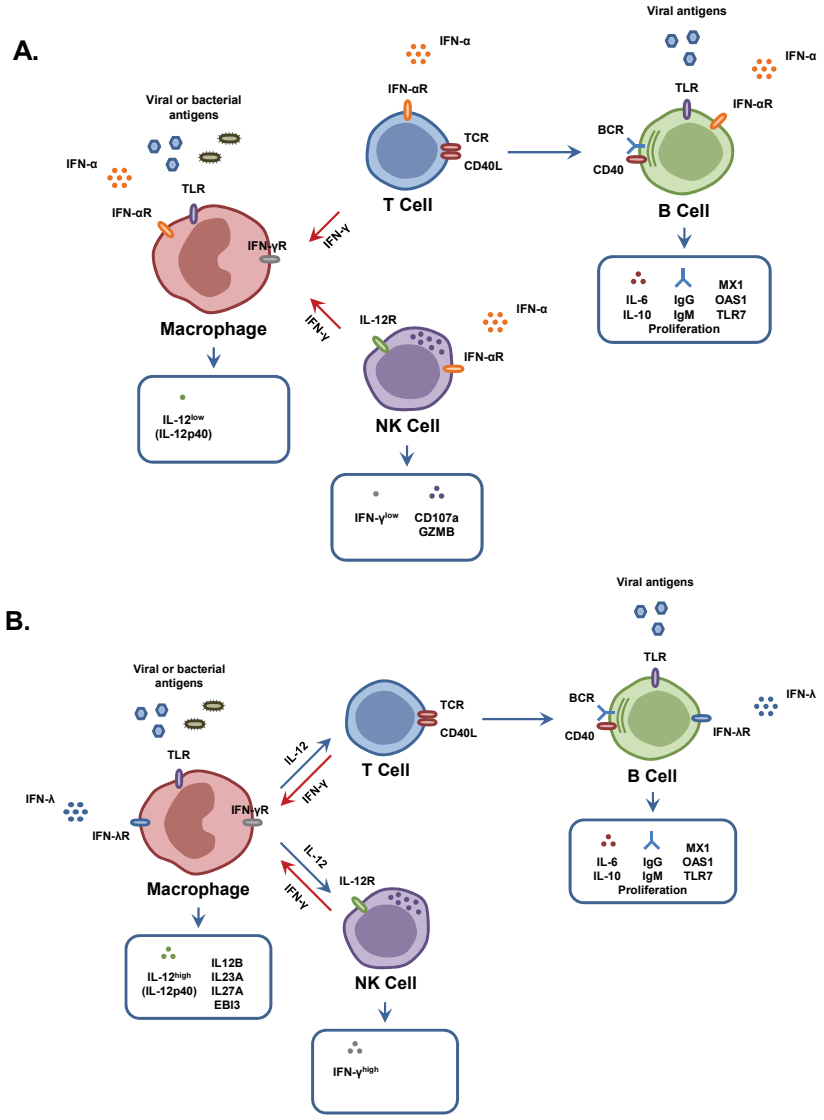


Figure 1. IFN-α and IFN-λ differentially regulate the innate immune response.

(A) IFN-α and (B) IFN-λ resemble each other in their ability to directly stimulate B cells and augment TLR-mediated cytokine (IL-6 and IL-10) and Ig production (IgG and IgM), partially attributed to an increase in TLR7 response and proliferation. IFN-λ alone is able to induce ISG (MX1 and OAS1) and TLR7 expression, but additional TLR and helper T cell signals (CD40 and BCR ligation) are required for B cell activation and induction of functional responses. Unlike IFN-α, IFN-λ is unable to directly promote effector function in NK cells (IFN-γ, CD107, and GZMB expression) due to the lack of IFN-λR1 expression. However, IFN-λ stimulation, in combination with TLR activation, is able to induce expression of select members of the IL-12 family of cytokines (IL12B, IL23A, IL27A, and EBI3) in monocyte-derived macrophages. In combination with IFN-γ priming, IFN-λ is therefore able to indirectly induce IFN-γ production by NK cells, and potentially CD8 T cells, through macrophage-mediated IL-12 production, ultimately creating an autocrine loop. This indirect macrophage-mediated effect is unique to type III IFNs, as stimulation with IFN-α reduces the IL-12p40 and bioactive IL-12 production of macrophages.

In Chapters 3 and 4, we demonstrate that IFN-λ is able to directly act on B cells and monocyte-derived macrophages, but not NK cells and monocytes, while IFN-α is able to directly modulate all these cell populations (Figure 1A-B). Specifically, both type I and III IFNs are able to augment TLR-mediated B cell activation, as well as cytokine (IL-6 and IL-10) and Ig production (IgG and IgM), partially attributed to an increase in TLR7 response and proliferation. Although IFN-λ alone is able to induce ISG (MX1 and OAS1) as well as TLR7 expression, additional TLR and helper T cell signals, CD40 and BCR ligation, are required for B cell activation and induction of functional responses. Unlike IFN-α, IFN-λ is unable to directly activate and promote effector function in NK cells (IFN-γ, CD107, and GZMB expression), presumably due to the lack of cellular expression of the IFN-λR1 component of the type III IFN receptor complex. However, IFN-λ priming, in combination with TLR activation, is able to induce expression of select members of the IL-12 family of cytokines (IL12B, IL23A, IL27A, and EBI3) in monocyte-derived macrophages. We further show that IFN-λ, in combination with IFN-γ, is able to indirectly affect NK cells through macrophage-mediated IL-12 production, ultimately inducing IFN-γ production and potentially creating an autocrine loop. This indirect macrophage-mediated effect is unique to type III IFNs, as stimulation with IFN-α reduced the IL-12p40 and bioactive IL-12 production of macrophages.

Although B cell activity is generally attributed to the antigen-specific adaptive immune response, B cells have also been shown to express and respond to non-specific antigens via PRRs such as TLR7. Additionally, exposure of B cells to type I IFNs strongly promotes humoral immunity via enhanced survival, reduced apoptosis, as well as increased B cell activation, proliferation, and Ig production.[20–22] In Chapter 3, we demonstrate that IFN-λ resembles IFN-α in all these aspects, specifically in regards to its upregulation of TLR7 expression and enhancement of subsequent responses. The enhancement of B cell responses, specifically the increase in antibody production we observe, by both type I and III IFNs could play an important part in the resolution of acute hepatitis infections. Individuals that spontaneously resolve HBV infection develop antibodies specific for HBsAg, and these HBsAg-specific antibodies have been shown to have virus-neutralizing activity and control HBV infection, even with the persistence of cccDNA.[23] Although the importance of neutralizing antibodies in the resolution of HCV infection is somewhat less evident, recent studies have described a role for HCV-specific antibodies in the control of infection.[24] In these studies, acute HCV resolution was associated with the early presence of neutralizing antibodies, whereas delayed development of neutralizing antibodies was observed in patients that eventually developed a chronic infection.[25,26] However, neutralizing antibodies specific for HCV E1 and E2 envelope proteins have been shown be susceptible to escape mutations.[27] This further stresses the importance of an immediate induction of antibody responses by B cells in the control and resolution

of acute HCV infection, before the development of escape mutations, potentially driven by a combination of innate immune, non-specific factors such as type I and III IFNs, as well as PAMPs.

Similar to that demonstrated for macrophages and NK cells in Chapter 4, IL-12-mediated cross-talk has been described between CD8 T cells and macrophages.[28] Reports describing effects of type III IFNs on T cells directly have been unconvincing,[29,30] with our own preliminary screenings performed during previous studies also failing to demonstrate T cell responses after exposure to IFN-λ alone or in combination with various co-stimuli (data not shown). This again implicates only an indirect role for IFN-λ in modulating CD8 T cell responses, that contrasts starkly to that of IFN-α, which has been described to directly regulate IFN-γ production by cytolytic T cells. These direct type I IFN effects have been shown to be both inducing as well as inhibiting in the production of IFN-γ by CD8 T cells and to have anti-proliferative effects on T cells.[31] If IFN-λ is able to indirectly enhance IFN-γ production of CD8 T cells, as we describe for NK cells, without the anti-proliferative effects of type I IFNs, this could provide distinct advantages in immune response to viral hepatitis infections. Effective IFN-γ production has been shown to be a critical factor in the inhibition of HBV and HCV replication and in the early response to hepatitis infections.[32–34] Additionally, the favoring of IL-12 production by IFN-λ, that we and others describe, also indicates a role for promoting type 1 over type 2 immune response and the induction of strong antiviral responses from type 1 helper T (T_H1) cells as well as CD8 T cells.[14,30,35] Collectively, via an alternative indirect route, IFN-λ has the ability to induce an anti-viral immune response by NK cells and cytolytic T cells, both required for an effective clearance of viral hepatitis infections.[36,37]

Conclusion and future perspectives

The ubiquitous expression of the IFN-α receptor across leukocyte populations (e.g. monocytes, NK cells, and T cells) and the ability of IFN-α to directly act on many of these cell types distinguishes it from IFN-λ. These features make IFN-α an integral and direct mediator in the induction of the immediate immune response to viral infection in the peripheral as well as the tissue compartments. In contrast, the restricted expression of the IFN-λ receptor prevents the direct action of IFN-λ on a significant proportion of leukocyte populations, with the exception of B cells, monocyte-differentiated macrophages, and pDCs. However, other studies have described effects of type III IFNs on tissue-resident cells, such as epithelial cells, hepatocytes, and synovial fibroblasts (discussed in Chapter 5).[38–40] The responses to IFN-λ have also been described to be kinetically distinct to those of IFN-α, with a more delayed and prolonged effect through activation of specific tissue-resident cells, such as hepatocytes.[15,41] These observations, in combination with the delayed indirect activation of NK cells by IFN-λ, mediated through

macrophage IL-12 production, could explain differences in biological functions described between the type I and type III IFNs in inflammatory responses, with a more tissue-specific (e.g intrahepatic) response induced type III IFNs. This localized induction of the immune response by IFN-λ could provide potential benefits over the aspecific, systemic effects of IFN-α, especially as an alternative therapeutic for viral hepatitis infections or other tissue-localized viral infections where DDAs are not available and/or patients are immune-compromised (e.g. hepatitis E virus (HEV) or hepatitis D virus (HDV)/HBV co-infection). Additionally, recent literature has shown that DAAs in HCV infection, although very highly efficacious and tolerable, may have some limitations in specific subpopulations that have a history of HCC, with surprisingly high rates of HCC reoccurrence being reported after SVR.[42] This has not been described for previous responders to IFN-α-based therapies, potentially due to the immunomodulatory properties of IFNs promoting not only antiviral, but also antitumor immunity. Therefore, the addition of IFN-λ to DAA therapy in specific subpopulations that have high grade fibrosis/cirrhosis, a high risk for HCC, and/or a history of HCC may help prevent the (re)occurrence of HCC, while providing a more tissue-specific immune response and less undesired adverse events than IFN-α.

THE PHENOTYPE AND FUNCTIONAL RESPONSES OF NK CELLS ARE ASSOCIATED WITH DISEASE STATE AND OUTCOME IN VIRAL HEPATITIS INFECTION

NK cells represent one of the first lines of defense of the innate immune response to viral hepatitis infections. Both the phenotype and function of NK cells have been extensively studied in chronic HBV and HCV infection, with studies reporting altered phenotype, cytokine production, and cytolytic capacity of NK cells in viral hepatitis patients.[37,43,44] However, many of these findings attributing various roles for NK cells in the pathogenesis of acute and chronic liver disease caused by HCV and HBV are controversial.[45] Therefore, in Chapters 6 and 7 we chose to address this issue by dissecting the heterogeneous chronic HBV and HCV populations into clinically relevant subgroups or by disease outcome, and analyzing specific phenotypic and function parameters of NK cells within these sub-groups.

NK cells in the natural history of chronic HBV infection

Blood transcriptome analysis of HBV patients identified NK cell and cytotoxicity related genes as distinctive markers in phases with elevated ALT levels, IA and ENEG, when compared to the IT phase.[46] To build on this study, in Chapter 6 we performed a detailed examination of blood NK cells throughout the course of the natural history of chronic HBV infection, specifically in regards to their

potential causal role in the fluctuations of liver damage markers and HBV replication observed during these clinical phases. The overall composition, phenotype, and cytolytic activity of the peripheral NK cell compartment remained relatively constant across all clinical phases, with the exception of a few specific markers (e.g. KIRs, NKp46, and CD57). However, the $CD56^{bright}$ NK cell population of chronic HBV patients differed in their ability to produce IFN-γ between the clinical phases pre- and post-HBeAg seroconversion. These results reject our original hypothesis that elevated levels of liver damage markers observed in the IA and ENEG clinical phases could be attributed to direct cytolytic killing of infected hepatocytes by NK cells, as no differences were observed in expression of the cytotoxic mediators perforin, granzyme B, and TRAIL across all clinical phases. This, however, does not rule a role for other cytotoxic immune cells, such as CD8+ T cells, in immune-mediated liver damage, although a recent study was unable to demonstrate distinct HBV-specific or global T cell activities in any of the clinical phases.[47] A potential limitation in this area of our study was that in order to provide a homogenous basis of comparison between groups, all patients with moderate to severe fibrosis or cirrhosis (fibrosis score > F2) were excluded. NK cells in patients with more advanced or severe fibrosis/cirrhosis could display differing phenotypic or functional profiles due to changes in intrahepatic factors (e.g. exposure to activated hepatitis stellate cells and altered cytokines micro-milieus).

Although the NK cell compartment composition and expression of cytolytic mediators did not differ between clinical phases, this does not exclude potential effects of other NK cell functions on the clinical manifestations observed in the natural history of HBV infection. The differential capacity to produce IFN-γ in $CD56^{bright}$ cells of chronic HBV patients pre- and post-HBeAg seroconversion, specifically between the IT/IA and IC phases, could be partially responsible for the changes in clinical parameters seen during the transition between these phases. IFN-γ has been shown to be a trigger for the process of non-cytolytic HBV clearance and recruitment of inflammatory immune cells in both the innate and adaptive immune response to HBV infection.[48,49] The increase in IFN-γ production we observe in $CD56^{bright}$ NK cells of IC patients could therefore be a driving factor in inhibiting HBV viral replication and a trigger for the subsequent immune responses eventually leading to HBsAg seroconversion and the viral control characteristic of this phase. The other possibility is that this differential production of IFN-γ observed could be the result of enhanced immune pressure due to high viral and antigen load present during the HBeAg-positive clinical phases, relieved after seroconversion and the subsequent viral suppression. However, we cannot say if this is a direct result of the high levels of viral particles and antigens present on NK cells or an indirect effect due to the coinciding immune activity in these phases. This data may also help to understand the impaired IFN-γ production described for NK cells of chronic HBV patients when compared to healthy controls, and suggests that this may not be a global phenomenon for chronic patients, but

only for those in specific phases with high viral pressure. This is corroborated by the recovery in IFN-γ production observed after anti-viral treatment and viral suppression, or blockade of specific immune cytokines observed in these studies.[50–52] Overall, this data shows that NK cell cytolysis is not directly responsible for initiating shifts in markers of liver damage observed in the progressing HBV clinical phases, but that these cells do play a role in the viral control characteristic of specific stages of the natural history of chronic HBV infection.

NK cell differentiation and memory in resolved and chronic HCV infections

Recent studies have demonstrated developmental and functional qualities for NK cells that are commonly associated with immunological memory, including the continuous differentiation and formation of specific NK cell repertoires in viral infection settings.[53–57] In Chapter 7, we assessed these phenotypic and functional parameters associated with NK cell differentiation and memory in patients three years after acute HCV infection and either the subsequent spontaneous clearance of infection or progression to chronicity. Although we were able to identify the $CD57^{+}NKG2C^{++}$ memory-like NK cell population, no significant differences were observed in the total percentages of this specific NK cell repertoire between uninfected, resolved, and chronic HCV patients, arguing against the hypothesis that the $CD57^{+}NKG2C^{++}$ NK cells observed are memory NK cells induced by HCV infection. Additionally, the protective features attributed to expanded expression of these populations upon re-exposure previously described in other viral infections, cannot justify the increased resistance to reinfection described in acute-resolved HCV patients. Our findings did, however, depict an altered, more extensively differentiated (increased KIR and CD57, as well as reduced NKG2A expression) NK cell compartment in patients after resolving an acute infection. The presence of a highly differentiated $KIR^{+}CD57^{+}NKG2A^{-}$ NK cell repertoire in acute HCV patients that clear infection is in line with previously described associations between the resolution of acute HCV infection and NK cell inhibitory receptors. Gene expression of KIRs, specifically KIR2DL3 and its human leukocyte antigen C group 1 (HLA-C1) ligand, have previously been linked to increased odd ratios of spontaneous HCV resolution and increased prevalence of $KIR2DL3^{+}NKG2A^{-}$ NK cells has been associated with protection from HCV infection in people who inject drugs.[58,59] This further suggests that presence of these differentiated NK cells, along with other innate immune system components, contribute to the viral containment and clearance in an acute HCV infection.

In contrast to patients that spontaneously resolve acute HCV infection, the NK cells of chronic HCV patients display a more naïve and effector phenotype (increased $CD56^{bright}$, NKp46, and IFN-γ expression) with elevated cytolytic capacities (granzyme B and TRAIL). NKp46 has been shown to be one of the principle avenues of NCR-mediated killing, with increased expression of this NCR being

correlated to the cytolytic activity of NK cells in HCV patients.[60] This supports the skewing toward cytotoxicity, with increased frequencies of granzyme B and TRAIL expressing cells, observed in chronic HCV patients. These phenotypic and functional traits of NK cells could be reflective of the persistent exposure to viral antigens and infected cells in a chronic disease setting. However, due to the retrospective nature of our study, and looking at patients three years after acute infection, we were unable to determine if these specific differences in NK cell compartments are the cause or result of the resolution of the acute infection or progression to chronicity. Future prospective studies, following individual patients with acute HCV infections could provide insight on this and potentially if baseline characteristics of or shifts in NK cell compartments are predictive of acute HCV infection outcome.

Conclusion and future perspectives

Although our initial hypotheses regarding the roles of NK cells in the varying HBV clinical phases as well as in the resolution of and protection against acute HCV (re)infection were proven incorrect, we were still able to demonstrate distinctive features and functions of NK cells that were linked to disease state and outcome. By dissecting the heterogeneous chronic HBV patient population into the four clinically distinct phases, we were able to assess aspects of NK cells in a more homogenous setting and investigate associations with specific disease parameters. The data presented in this thesis suggests that NK cells are not globally impaired both in their ability to produce IFN-γ and other facets, but instead that functional differences exist in specific NK cell subsets pre- and post-HBeAg seroconversion. The therapeutic response rates to IFN-α and likelihood of HBsAg conversion are significantly higher in HBeAg-positive patients, suggesting an underlying difference of immune mechanisms between HBeAg-negative and -positive disease. As IFN-α has been shown to be a potent regulator of NK cells, the phenotypic and functional shifts we describe could be one of these underlying factors dictating the differences in HBV patient treatment outcomes. Lastly, although memory-like NK cells have been associated with protection upon re-exposure of other viral infections, such as CMV, this does not appear to be the case for HCV. Why this differs remains unclear, but differences in viral antigens, tropism, other immune responses and cytokine milieus could have an effect on the differentiation and development of a memory-like NK cell population.

Lastly, the work in this thesis, and in the majority of other published studies, is focused on the peripheral compartment and understanding the systemic effects of viral hepatitis on NK cells and the immune system. This completely neglects the intrahepatic compartment, where NK cells represent one of the most abundant lymphocyte populations and highly specialized liver-resident NK cells have been described. These observations, in combination with the unique microenvironment

of the liver, make it imperative that future studies, although difficult, investigate the role of intrahepatic NK cells on specific disease parameters, such as ALT and fibrosis, as well as in the resolution of or progression to chronicity of viral hepatitis infections.

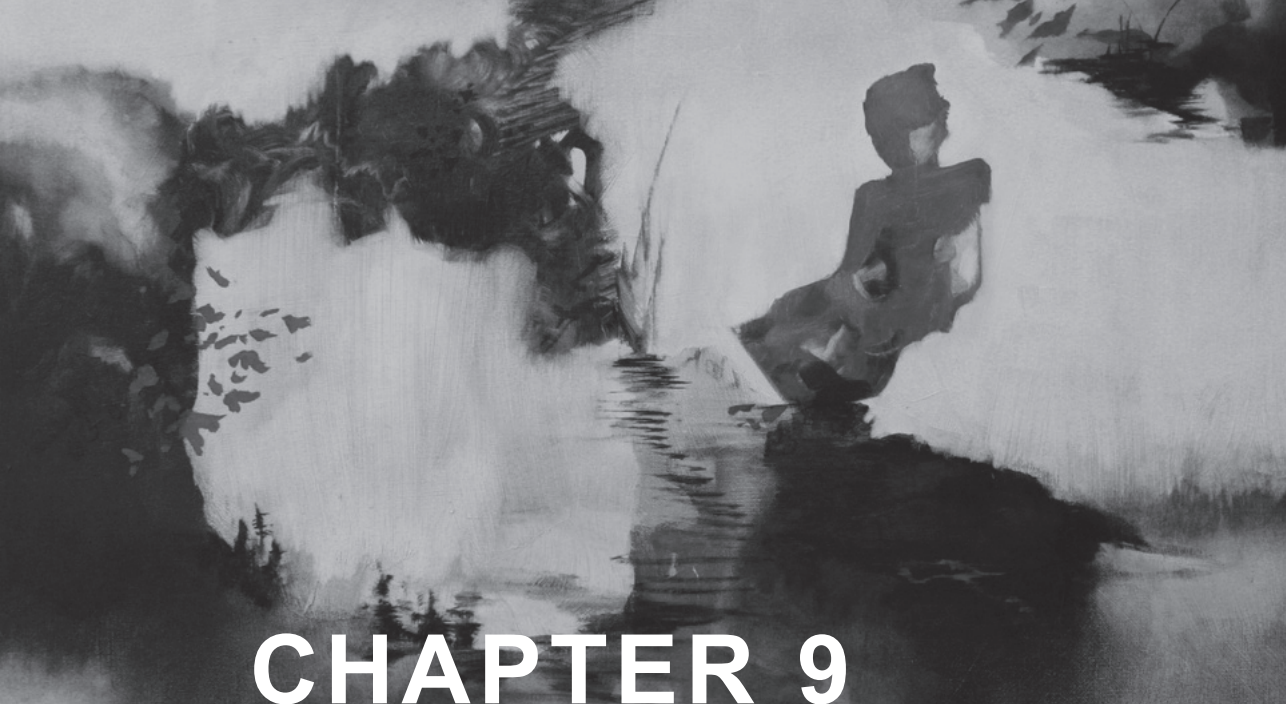
REFERENCES

1. World Health Organization (WHO). Hepatitis B Fact Sheet. (2016). Available at: http://www.who.int/mediacentre/factsheets/fs204/en/.
2. World Health Organization (WHO). Hepatitis. (2016). Available at: http://www.who.int/hepatitis/en/.
3. World Health Organization (WHO). Hepatitis C Fact Sheet. (2016). Available at: http://www.who.int/mediacentre/factsheets/fs164/en/.
4. Ge, D. *et al.* Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* **461,** 399–401 (2009).
5. Suppiah, V. *et al.* IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat. Genet.* **41,** 1100–4 (2009).
6. Tanaka, Y. *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat. Genet.* **41,** 1105–9 (2009).
7. Thomas, D. L. *et al.* Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* **461,** 798–801 (2009).
8. Prokunina-Olsson, L. *et al.* A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nat. Genet.* **45,** 164–71 (2013).
9. Park, H. *et al.* IL-29 is the dominant type III interferon produced by hepatocytes during acute hepatitis C virus infection. *Hepatology* **56,** 2060–70 (2012).
10. Yin, Z. *et al.* Type III IFNs are produced by and stimulate human plasmacytoid dendritic cells. *J. Immunol.* **189,** 2735–45 (2012).
11. Yoshio, S. *et al.* Human blood dendritic cell antigen 3 (BDCA3)(+) dendritic cells are a potent producer of interferon-λ in response to hepatitis C virus. *Hepatology* **57,** 1705–15 (2013).
12. Chandra, P. K. *et al.* HCV infection selectively impairs type I but not type III IFN signaling. *Am. J. Pathol.* **184,** 214–29 (2014).
13. Friborg, J. *et al.* Impairment of type I but not type III IFN signaling by hepatitis C virus infection influences antiviral responses in primary human hepatocytes. *PLoS One* **10,** e0121734 (2015).
14. Liu, B.-S., Janssen, H. L. A. & Boonstra, A. IL-29 and IFN-α differ in their ability to modulate IL-12 production by TLR-activated human macrophages and exhibit differential regulation of the IFNγ receptor expression. *Blood* **117,** 2385–95 (2011).
15. Jilg, N. *et al.* Kinetic differences in the induction of interferon stimulated genes by interferon-α and interleukin 28B are altered by infection with hepatitis C virus. *Hepatology* **59,** 1250–61 (2014).
16. Urban, T. J. *et al.* IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. *Hepatology* **52,** 1888–96 (2010).
17. Freeman, J. *et al.* Pegylated interferons Lambda-1a and alfa-2a display different gene induction and cytokine and chemokine release profiles in whole blood, human hepatocytes and peripheral blood mononuclear cells. *J. Viral Hepat.* **21,** e1-9 (2014).

18. Sonneveld, M. J., Brouwer, W. P. & Janssen, H. L. A. Studies of *IL28B* genotype and response to peginterferon in chronic hepatitis B should be stratified by HBV genotype. *Hepatology* **57,** 1283–1283 (2013).
19. Sonneveld, M. J. *et al.* Polymorphisms Near IL28B and Serologic Response to Peginterferon in HBeAg-Positive Patients With Chronic Hepatitis B. *Gastroenterology* **142,** 513–520.e1 (2012).
20. Le Bon, A. *et al.* Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. *Immunity* **14,** 461–70 (2001).
21. Le Bon, A. *et al.* Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. *J. Immunol.* **176,** 2074–8 (2006).
22. Swanson, C. L. *et al.* Type I IFN enhances follicular B cell contribution to the T cell-independent antibody response. *J. Exp. Med.* **207,** 1485–500 (2010).
23. Guidotti, L. G. & Chisari, F. V. Immunobiology and pathogenesis of viral hepatitis. *Annu. Rev. Pathol.* **1,** 23–61 (2006).
24. Cashman, S. B., Marsden, B. D. & Dustin, L. B. The Humoral Immune Response to HCV: Understanding is Key to Vaccine Development. *Front. Immunol.* **5,** 550 (2014).
25. Esteban-Riesco, L. *et al.* Rapid and sustained autologous neutralizing response leading to early spontaneous recovery after HCV infection. *Virology* **444,** 90–9 (2013).
26. Osburn, W. O. *et al.* Clearance of hepatitis C infection is associated with the early appearance of broad neutralizing antibody responses. *Hepatology* **59,** 2140–2151 (2014).
27. Dowd, K. A., Netski, D. M., Wang, X., Cox, A. L. & Ray, S. C. Selection Pressure From Neutralizing Antibodies Drives Sequence Evolution During Acute Infection With Hepatitis C Virus. *Gastroenterology* **136,** 2377–2386 (2009).
28. Biswas, S. K. & Mantovani, A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat. Immunol.* **11,** 889–896 (2010).
29. Dai, J., Megjugorac, N. J., Gallagher, G. E., Yu, R. Y. L. & Gallagher, G. responses in human naive and memory T cells IFN- 1 (IL-29) inhibits GATA3 expression and suppresses Th2 responses in human naive and memory T cells. *Blood* **1,** 5829–5838 (2009).
30. Egli, A. *et al.* IL-28B is a Key Regulator of B- and T-Cell Vaccine Responses against Influenza. *PLoS Pathog.* **10,** (2014).
31. McNab, F., Mayer-Barber, K., Sher, A., Wack, A. & O'Garra, A. Type I interferons in infectious disease. TL - 15. *Nat. Rev. Immunol.* **15 VN-r,** 87–103 (2015).
32. Kokordelis, P. *et al.* An effective interferon-gamma-mediated inhibition of hepatitis C virus replication by natural killer cells is associated with spontaneous clearance of acute hepatitis C in human immunodeficiency virus-positive patients. *Hepatology* **59,** 814–827 (2014).
33. Kakimi, K., Guidotti, L. G., Koezuka, Y. & Chisari, F. V. Natural killer T cell activation inhibits hepatitis B virus replication in vivo. *J. Exp. Med.* **192,** 921–30 (2000).
34. Biron, C. A., Nguyen, K. B., Pien, G. C., Cousens, L. P. & Salazar-Mather, T. P. NATURAL KILLER CELLS IN ANTIVIRAL DEFENSE: Function and Regulation by Innate Cytokines. *Annu. Rev. Immunol.* **17,** 189–220 (1999).
35. Koltsida, O. *et al.* IL-28A (IFN-λ2) modulates lung DC function to promote Th1 immune skewing and suppress allergic airway disease. *EMBO Mol. Med.* **3,** 348–61 (2011).

36. Rehermann, B. & Nascimbeni, M. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat. Rev. Immunol.* **5,** 215–229 (2005).
37. Rehermann, B. Natural Killer Cells in Viral Hepatitis. *C. Cell. Mol. Gastroenterol. Hepatol.* **1,** 578–588 (2015).
38. Xu, L. *et al.* IL-29 enhances Toll-like receptor-mediated IL-6 and IL-8 production by the synovial fibroblasts from rheumatoid arthritis patients. *Arthritis Res. Ther.* **15,** R170 (2013).
39. Doyle, S. E. *et al.* Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. *Hepatology* **44,** 896–906 (2006).
40. Sommereyns, C., Paul, S., Staeheli, P. & Michiels, T. IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. *PLoS Pathog.* **4,** e1000017 (2008).
41. Olagnier, D. & Hiscott, J. Type I and type III interferon-induced immune response: it's a matter of kinetics and magnitude. *Hepatology* **59,** 1225–8 (2014).
42. Reig, M. *et al.* Unexpected early tumor recurrence in patients with hepatitis C virus -related hepatocellular carcinoma undergoing interferon-free therapy: a note of caution. *J. Hepatol.* (2016). doi:10.1016/j.jhep.2016.04.008
43. Lunemann, S. *et al.* Compromised Function of Natural Killer Cells in Acute and Chronic Viral Hepatitis. **209,** (2014).
44. Oliviero, B. *et al.* Natural Killer Cell Functional Dichotomy in Chronic Hepatitis B and Chronic Hepatitis C Virus Infections. *YGAST* **137,** 1151–1160.e7 (2009).
45. Mondelli, M. U., Varchetta, S. & Oliviero, B. Natural killer cells in viral hepatitis: facts and controversies. *Eur. J. Clin. Invest.* **40,** 851–863 (2010).
46. Vanwolleghem, T. *et al.* Re-evaluation of hepatitis B virus clinical phases by systems biology identifies unappreciated roles for the innate immune response and B cells. *Hepatology* **62,** 87–100 (2015).
47. Park, J. J. *et al.* Hepatitis B Virus-Specific and Global T-Cell Dysfunction in Chronic Hepatitis B. *Gastroenterology* **150,** 684–695e5 (2016).
48. Maini, M. K. & Gehring, A. J. The role of innate immunity in the immunopathology and treatment of HBV infection. *J. Hepatol.* **64,** S60-70 (2016).
49. Bertoletti, A. & Ferrari, C. Adaptive immunity in HBV infection. *J. Hepatol.* **64,** S71–S83 (2016).
50. Tjwa, E. T. T. L., Oord, G. W. Van, Hegmans, J. P., Janssen, H. L. A. & Woltman, A. M. Viral load reduction improves activation and function of natural killer cells in patients with chronic hepatitis B. *J. Hepatol.* **54,** 209–218 (2011).
51. Peppa, D. *et al.* Blockade of Immunosuppressive Cytokines Restores NK Cell Antiviral Function in Chronic Hepatitis B Virus Infection. **6,** (2010).
52. Boni, C. *et al.* Natural Killer Cell Phenotype Modulation and Natural Killer/T-Cell Interplay in Nucleos(t)ide Analogue-Treated Hepatitis e Antigen-Negative Patients With Chronic Hepatitis B. 1697–1709 (2015). doi:10.1002/hep.28155
53. Björkström, N. K. *et al.* Rapid expansion and long-term persistence of elevated NK cell numbers in humans infected with hantavirus. *J. Exp. Med.* **208,** 13–21 (2011).
54. Béziat, V. *et al.* CMV drives clonal expansion of NKG2C+ NK cells expressing self-specific KIRs in chronic hepatitis patients. *Eur. J. Immunol.* **42,** 447–57 (2012).

55. Lopez-Vergès, S. *et al.* Expansion of a unique CD57^{+}NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 14725–32 (2011).
56. Sun, J. C., Lopez-Verges, S., Kim, C. C., DeRisi, J. L. & Lanier, L. L. NK cells and immune "memory". *J. Immunol.* **186,** 1891–7 (2011).
57. O'Sullivan, T. E., Sun, J. C. & Lanier, L. L. Natural Killer Cell Memory. *Immunity* **43,** 634–45 (2015).
58. Khakoo, S. I. *et al.* HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* **305,** 872–4 (2004).
59. Thoens, C. *et al.* KIR2DL3^{+}NKG2A^{-} natural killer cells are associated with protection from productive hepatitis C virus infection in people who inject drugs. *J. Hepatol.* **61,** 475–81 (2014).
60. Pembroke, T. *et al.* The paradox of NKp46 + natural killer cells : drivers of severe hepatitis C virus-induced pathology but in-vivo resistance to interferon α treatment. 515–524 (2014). doi:10.1136/gutjnl-2013-304472



CHAPTER 9

NEDERLANDSE SAMENVATTING

Hepatitis B virus (HBV) en hepatitis C virus (HCV) infecties zijn wereldwijd de twee voornaamste oorzaken van chronische ontsteking van de lever. Ongeveer 370–390 miljoen mensen lijden eraan. Op dit moment worden de virale en immunologische mechanismen die de basis vormen van zowel de chronische infectie van virale hepatitis als de genezing niet volledig begrepen. Onderzoek naar en identificatie van de integrale componenten in de immuun reacties van de patiënt die cumulatief leiden tot de ontwikkeling van een chronische infectie of tot een effectieve uitroeiing van het virus zijn van het grootste belang. Het algemene doel van deze thesis is daarom het begrijpen van de functie, en dysfunctie, van specifieke aspecten van het aangeboren immuunsysteem in de aanhoudendheid en/of de genezing van virale hepatitis infectie. De twee voornaamste componenten van de immuunrespons waarop in deze thesis het focus ligt zijn de rollen die interferon (IFNs) en natural killer (NK) cellen spelen in het bepalen van de staat van de ziekte en het resultaat van infectie.

SYSTEMISCHE WAARDEN EN IMMUNOLOGISCHE REGULATIE VAN TYPE III IFNS IN VIRALE HEPATITIS INFECTIE

IFNs zijn een van de basis componenten van de aangeboren immuunrespons op virale infecties en zijn voor vele jaren een belangrijke hoeksteen in de behandeling van chronische HCV en HBV infectie geweest tot aan de recente introductie van direct-acting antivirals (DAAs). Deze klasse van cytokines is beschreven als hebbende een antivirale, antiproliferatieve, en immunomodulerende uitwerking, en is verdeeld in drie grote subklassen; type I, II, en III IFNs, elk met unieke complementaire receptor complexen waardoor zij signaleren. Echter, in tegenstelling tot de hoog prominente type I IFNs (bijv. IFN-α), kregen type III IFNs, opgemaakt uit IFN-λ1, IFN-λ2, IFN-λ3, en IFN-λ4, relatief weinig aandacht tot de ontdekking van polymorfismen binnen de IFN-λ gen die geassocieerd zijn met spontane zowel als met IFN-α/ribavirin therapie geassocieerde genezing van HCV infectie. Alle leden van de IFN-λ familie gebruiken een specifiek transmembraan receptor heterodimer, het IFN-αR1 en IL-10R2 complex, dat structureel verschilt van het IFN-α receptor complex, een heterodimer van de IFN-αR1 en IFN-αR2 moleculen. In tegenstelling tot de alomtegenwoordige IFN-α receptor is de distributie van de IFN-λ receptor meer gelimiteerd. Type I IFNs blijken sterke modulatoren van meerdere immuun cellen populaties te zijn, inclusief antigeen–presenterende cellen zoals dendritische cellen (DCs), monocyten en macrofagen (en hun stimulatie van T-cel responses) zowel als B cellen en NK cellen. Echter, ten gevolge van de verschillen in IFN-α en IFN-λ receptor prevalentie en hun respectievelijke signalering kinetiek, blijven de effecten van IFN-λ op immuun cellen nog controversieel met beperkt bewijs voor veel van deze populaties.

De associatie tussen polymorfismen binnen het IFN-λ gen locus en genezing van HCV infectie wordt nog slecht begrepen, maar suggereert dat IFN-λ een belangrijke rol speelt in het tot stand komen van een effectieve antivirale respons. Daarom was de eerst logische stap in het bepalen van het belang van IFN-λ in virale hepatitis het onderzoeken van endogene waarden in het serum van chronisch geïnfecteerde patiënten en hoe die geassocieerd zijn met patiënt IFN-λ polymorfisme genotypen zowel als ziekte status/uitkomst in HBV en HCV infectie. In **Hoofdstuk 2** hebben we de waarden van IFN-λ1 en IFN-λ2/3 gemeten in het serum van gezonde controle personen, chronische HCV patiënten, patiënten die genezen zijn van HCV infectie, chronisch geïnfecteerde HBV patiënten, acuut geinfecteerde HBV patiënten en chronische HBV patiënten die op dit moment DAA therapie krijgen. We laten zien dat serum IFN-λ waarden niet worden beïnvloed door infectie met zowel HCV of HBV, en dat IFN-λ waarden niet geassocieerd zijn met specifieke ziekte stadia, de uitkomst van het ziekteproces of andere parameters (bijv. ALT en IFN-λ3 rs12979860 genotype).

Naast de rol van endogeen geproduceerd IFN-λ in de setting van chronische virale hepatitis, blijft ook veel van de onderliggende basale biologie van IFN-λ onverklaard. De antivirale aspecten van deze klasse cytokines zijn welomschreven, met vele overeenkomsten met die toegeschreven aan type I IFNs. Dit brengt dan echter de vraag op wat het evolutionaire voordeel van het hebben van twee klassen IFNs als hun immunologische rol inderdaad hetzelfde is? Daarom is er meer onderzoek nodig t.a.v. de weinige onderscheidingen beschreven voor type III IFNs, met name de IFN-λ receptor en diens expressie op immuun cellen zowel als op de geassocieerde responsen na stimulatie. Omdat er studies waren die de expressie van de IFN-λ receptor op B cellen rapporteren, maar met beperkt beschikbare informatie over de werkelijke effecten van IFN-λ, besloten we om de rol van IFN-λ in de regulatie en modulatie van menselijke B cell functie in detail te bestuderen, en die te vergelijken met die van IFN-α.

In **Hoofdstuk 3** laten we zien dat zowel type I en III IFNs in staat zijn om zowel toll-like receptors (TLR)-gemedieerde B cellen activatie als cytokine (IL-6 en IL10) en immunoglobuline productie (lgG en lgM) te verbeteren. Dit was ten dele toegeschreven aan een verhoging in TLR7 respons en proliferatie. Hoewel IFN-λ alleen in staat is om IFN-stimulated genes (ISGs; MX1 en OAS1) zowel als TLR7 expressie teweeg te brengen, zijn er aanvullende TLR en helper T cel signalen, CD40 en BCR ligatie nodig voor B cel activatie en het opwekken van functionele responsen.

NK cellen vertegenwoordigen een andere groep van lymfocyten met slecht beschreven respons op type III IFNs; er zijn tegenstrijdige resultaten gerapporteerd voor zowel de expressie van de IFN-λ receptor als de directe effecten van IFN-λ op menselijke NK cellen. Studies hebben aangetoond dat monocyt-afgeleide macrofagen de IFN receptor expressen en tevens deze cellen de productie van IL-12p40 verhogen als deze receptor geactiveerd wordt, met name in combinatie

met activatie via TLRs. Dit is vooral van belang in hepatitis infectie omdat TLR-geactiveerde macrofagen van de lever een effector functie blijken uit te oefenen in NK cellen, door tussenkomst van cytokine-geïnduceerde activatie en productie van IFN-λ.

Om de biologische rol van IFN-λ daarom beter te kunnen begrijpen hebben we de effecten van IFN-λ op NK cellen in zowel een directe en indirect capaciteit door zijn modulatie van TLR-geactiveerde macrofagen onderzocht (**Hoofdstuk 4**). In dit hoofdstuk laten we zien dat in tegenstelling tot IFN-α, IFN-λ niet in staat is om de effector functie in NK cellen (IFN-γ, CD107a, en GZMB expressie) direct te activeren en stimuleren, waarschijnlijk door het gebrek aan cellulaire expressie van de IFN-λR1 molecuul van het type III IFN receptor complex. Echter, IFN-λ priming, in combinatie met TLR activatie, is in staat om expressie van selecte leden van de IL-12 familie van cytokines (IL12B, IL23A, IL27A, en EB13) te induceren in monocyt-afgeleide macrofagen. Overeenkomstig hebben andere studies de effecten van IFN-λ op het moduleren van de functie van TLR-geactiveerde fibroblasten onderzocht, met name IL-6 en IL-8 productie, in het kader van reumatoïde artritis synoviale ontsteking. Onze commentaren op deze studies en het belang van IFN-λ in het kader van chronische ontstekings ziekten zoals reumatoïde artritis worden besproken in **Hoofdstuk 5**.

Conclusies IFNs

De alomtegenwoordige expressie van de IFN-α receptor over leukocyten populaties (bijv. monocyten, NK cellen en T cellen) en het vermogen van IFN-α om direct op deze cel typen te werken onderscheidt het van IFN-λ. Deze kenmerken maken IFN-α een integrale en directe mediator in de inductie van de onmiddellijke immuun respons op virale infectie zowel in de perifere als de weefsel compartimenten. In tegenstelling hiertoe belet de beperkte expressie van de IFN-λ receptor de directe actie van IFN-λ op een aanzienlijk deel van de leukocyten populaties, met uitzondering van B cellen, monocyt-afgeleide macrofagen, en plasmacytoid DCs.

Type III IFNs echter zijn in staat om te werken op weefsel-resident cellen, zoals epitheel cellen, hepatocyten, en synoviale fibroblasten. Deze lokale inductie van de immuun respons door IFN-λ zou mogelijke voordelen kunnen bieden ten opzichte van de aspecifieke, systemische effecten van IFN-α, vooral als een alternatieve therapie voor virale hepatische infecties of andere weefsel-gelokaliseerde virale infecties waar DAAs niet beschikbaar zijn en/of bij patiënten met een verzwakt immuun system (bijv. hepatitis E virus) of hepatitis delta virus/HBV co-infectie). Verder heeft recente literatuur laten zien dat DAAs in HCV infectie, hoewel zeer effectief en goed te verdragen, mogelijk enige beperkingen zou kunnen hebben in specifieke subpopulaties zoals patiënten met de ziekte-geschiedenis hepatocellular carcinoma (HCC), waarbij een verrassend hoog recidief HCC gerapporteerd wordt na sustained virological response (SVR). Dit

is niet beschreven voor eerdere responderen op IFN-α-gebaseerde therapieën, mogelijk door de immunomodulerende eigenschappen van IFNs die niet alleen antivirale, maar ook antitumor immuniteit bevorderen. Daarom kan de toevoeging van IFN-λ aan DAA therapie in specifieke subpopulaties die een hoge graad van fibrose/cirrose, een hoog risico voor HCC, en/of bekend zijn met HCC mogelijk een recidief van HCC helpen voorkomen, terwijl het een meer weefselspecifieke respons en minder ongewenste schadelijke effecten geeft dan IFN-α.

DE FENOTYPE EN FUNCTIONELE RESPONSE VAN NK CELLEN ZIJN GEASSOCIEERD MET STAAT VAN DE ZIEKTE EN AFLOOP VAN DE VIRALE HEPATITIS INFECTIE

NK cellen vertegenwoordigen een van de eerste verdediging linies van de natuurlijke immuun respons op virale infecties. Geactiveerde NK cellen reageren op stress signalen van met virus geïnfecteerde cellen door middel van twee typen mechanismen; directe mechanismen, zoals vrijgave en expressie van cytotoxische mediators als granzymes, perforins, en tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), dat resulteert in lysis van de geïnfecteerde cellen, en indirecte mechanismen die de afgifte van cytokines zoals IFN-λ en TNF omvatten, en antivirale responsen oproepen die virale replicatie remmen. Deze door stress op gang gebrachte signalen bestaan uit oppervlakte markers, zoals gemodificeerde major histocompatibility complex (MHC) expressie en liganden op regulerende receptoren zowel als ongebonden factoren. NK cel activiteit wordt alleen geregeld door een evenwicht van activerende en remmende receptoren op het cel oppervlak (bijv. C-type lectin-like receptoren; natural cytotoxicity receptors [NCRs] en killer Ig-like receptors [KIRs]). Tijdens een virale infectie verschuift het evenwicht van remming naar activering nadat de kritieke drempel van activatie signalen het wint van de drempel van remming signalen. Zowel het fenotype en de functie van NK cellen zijn grondig bestudeerd in chronische HBV en HCV infectie, met studies die gewijzigd fenotype, cytokine productie, en cytologische capaciteit van NK cellen in patiënten met virale hepatitis rapporteren. Echter, veel van deze resultaten, die NK cellen verschillende rollen in de pathogenese van acute en chronische leverziekte veroorzaakt door HCV en HBV toeschrijven, zijn controversieel. Daarom hebben we ervoor gekozen om in de **Hoofdstukken 6 en 7** dit onderwerp te bespreken door de heterogene chronische HBV en HCV populaties te ontleden in klinisch relevante subgroepen of door ziekte uitkomst, en specifieke fenotypische en functie parameters van NK cellen binnen deze subgroepen te analyseren.

Chronische HBV infectie wordt gekarakteriseerd door ziekte episodes met verschillende virale titers en/of tekenen van lever beschadiging (bijv. ALT). Een eerdere systems-biologie studie van de perifere bloed transcriptomes in de ver-

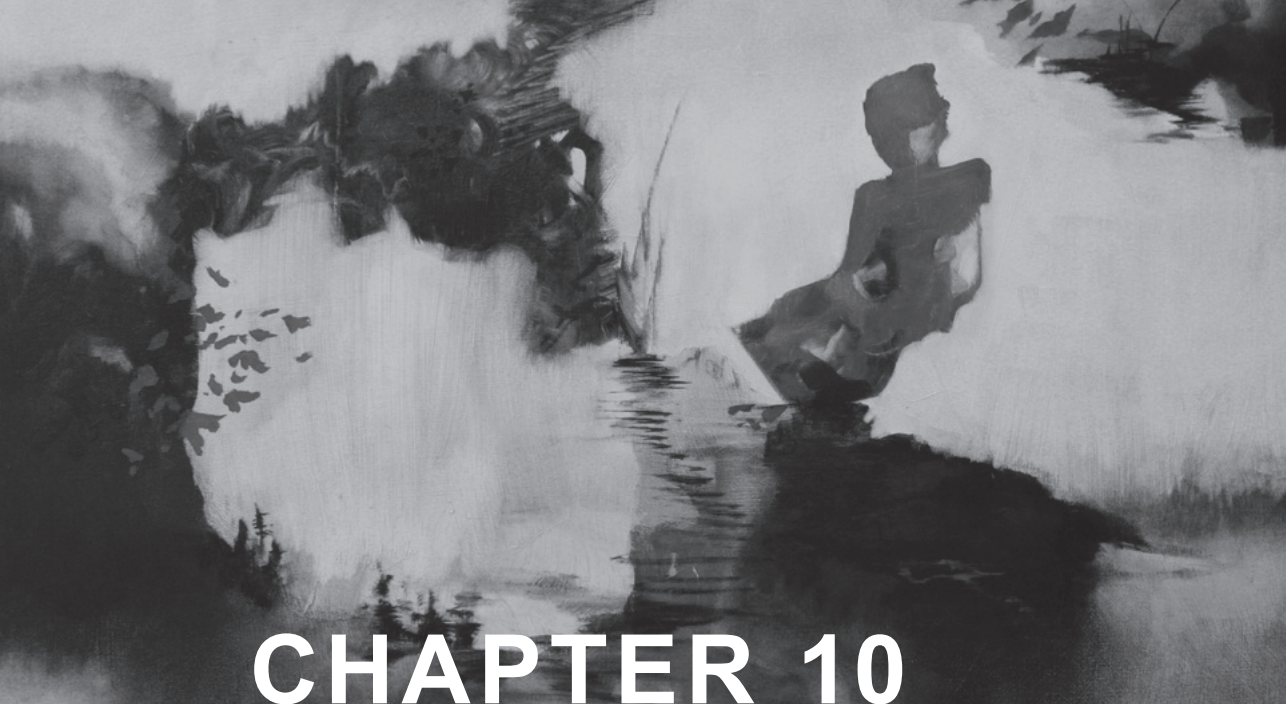
scheidene chronische HBV klinische fasen rapporteerde een upregulatie van cytotoxiciteit/NK cel activiteit-gerelateerde genen geclusterd in de HBeAg-positive immune active (IA) en HBeAg-negative hepatitis (ENEG) fasen, mogelijk duidend op een link tussen NK cel activiteit en de verhoging in ALT die gezien wordt in deze fasen. We hebben er daarom voor gekozen om de rol van differentiaal NK cel functionaliteiten in het bijdragen aan de onderscheidende kenmerken die gezien worden tijdens de HBV klinische fasen, met inbegrip van de fluctuaties in lever toxiciteit en HBV replicatie te bestuderen. Daarom hebben we in **Hoofdstuk 6** een gedetailleerd onderzoek van bloed NK cellen gedurende het natuurlijke verloop van chronische HBV infectie gedaan, in het bijzonder met betrekking tot hun mogelijke causale rol in de fluctuaties van lever toxiciteit en HBV replicatie die gezien wordt tijdens deze klinische fasen. De algemene samenstelling, fenotype, en cytolytische activiteit van het perifere NK cel compartiment bleef relatief constant gedurende alle klinische fasen, met uitzondering van een paar specifieke markers (bijv. KIRs, NKp46, en CD57). Echter, de $CD56^{bright}$ NK cel populatie van chronische HBV patiënten verschilde in hun vermogen om IFN-λ te produceren tussen de klinische fasen pre- en post-HBeAg seroconversie. Deze resultaten verwerpen onze oorspronkelijke hypothese dat verhoogde waarden van lever enzymen gezien in de IA en ENEG klinische fasen toegeschreven zouden kunnen worden aan direct cytolytische doden van geïnfecteerde hepatocyten door NK cellen, omdat er geen verschillen werden gezien in expressie van de cytotoxische mediators perforin, granzyme B en TRAIL over alle klinische fasen.

De hoge graad van spontane genezing gezien in patiënten waarbij eerder een acute HCV infectie is verdwenen wijzen er in sterke mate op dat immunologisch geheugen, en daarna immuniteit verkregen tijdens de primaire infectie een rol zou kunnen spelen in de bescherming tegen HCV re-infectie. Recente studies hebben ontwikkelende en functionele kwaliteiten voor NK cellen beschreven die veelal geassocieerd worden met immunologisch geheugen, met inbegrip van de voortdurende differentiatie en formatie van specifieke NK cellen repertoires tijdens virale infectie. Met beperkt beschikbare informatie over de ontwikkeling en persistentie van memory-like NK cellen gedurende en na HCV infectie, hebben we ervoor gekozen om in **Hoofdstuk 7** de NK cel compartimenten van individuele personen die een HCV infectie hebben overwonnen te karakteriseren en te vergelijken met die van individuele personen die een chronische infectie hebben ontwikkeld. In dit hoofdstuk hebben we de fenotypische en functionele parameters die geassocieerd zijn met NK cel differentiatie en geheugen onderzocht in patiënten drie jaar na acute HCV infectie en ofwel de daaropvolgende spontane genezing of de ontwikkeling tot chronische infectie. Hoewel we in staat waren om de $CD57^{+}NKG2C^{++}$ memory-like cellen populaties te identificeren, werden er geen significante verschillen gezien in de totale percentages van dit specifieke NK cel repertoire tussen de niet-geïnfecteerde, genezen, en chronische HCV patiënten; dit verwerpt de hypothese dat de geobserveerde $CD57^{+}NKG2C^{++}$ NK

cellen memory NK cellen zijn die geïnduceerd zijn door HCV infectie . Verder kunnen de beschermende kenmerken die toegeschreven werden aan de uitgebreide expressie van deze populaties bij hernieuwde blootstelling eerder beschreven in andere virale infecties niet de verhoogde resistentie tegen re-infectie beschreven in acuut-genezen HCV patiënten verklaren. Onze bevindingen geven echter wel een veranderd, meer uitgebreid gedifferentieerd (verhoogd KIR en CD57, zowel als verminderd NKG2A expressie) NK cel compartiment te zien in patiënten na een acuut genezen infectie. In tegenstelling tot de acute-genezen patiënten, hebben de NK cellen van chronische HCV patiënten een meer naïef en effector fenotype, met verhoogd $CD56^{bright}$, NKp46, en IFN-γ expressie, zo wel als verhoogde cytolytische capaciteiten (granzyme B en TRAIL).

Conclusies NK cellen

Alhoewel onze aanvankelijke hypotheses met betrekking tot de rol van NK cellen in de verschillende HBV klinische fasen zowel als in de genezing van en bescherming tegen acute HCV (re)infectie onjuist bleken te zijn, zijn we er wel toe in staat gebleken om de kenmerkende eigenschappen en functies van NK cellen die gekoppeld waren aan de staat van de ziekte en eventuele afloop te definiëren. Door het ontleden van de heterogene chronische HBV patiënten populatie in vier klinisch kenmerkende fasen zijn we in staat geweest om aspecten van NK cellen in een meer homogene setting te benaderen en om associaties met specifieke ziekte parameters te onderzoeken. De in deze thesis gepresenteerde data suggereren dat NK cellen in HBV patiënten over het algemeen niet aangetast zijn in zowel hun vermogen om IFN-γ en andere factoren te produceren, maar in plaats daarvan dat er functionele verschillen bestaan in specifieke NK cellen subsets pre- en post-HBeAg seroconversie. De therapeutische responsen met betrekking tot IFN-α en de waarschijnlijkheid van HBsAg seroconversie zijn beduidend in HBeAg positieve patiënten, duidend op een onderliggend verschil in immuun mechanismen tussen HBeAg-negatieve en-positieve ziekte. Omdat het is aangetoond dat IFN-α een sterke regulator van NK cellen is, zouden de fenotypische en functionele veranderingen die wij beschrijven een van deze onderliggende factoren kunnen zijn die de verschillen in behandeling uitkomst in HBV sturen. Ten laatste, hoewel memory-like NK cellen geassocieerd zijn met bescherming bij re-exposure in andere virale infecties zoals cytomegalovirus (CMV), lijkt dit niet het geval te zijn voor HCV. Waarom dit anders is blijft onduidelijk, maar verschillen in virale antigenen, tropisme, andere immuun responses en cytokine milieus zouden een effect kunnen hebben op de differentiatie en ontwikkeling van een memory-like NK cel populatie. Verder inzicht in de rol die NK cellen spelen in de progressie van de ziekte en de uiteindelijke afloop van virale hepatitis is van belang, met name om deze en andere natuurlijke immuun cellen te manipuleren met als doel om hun activiteit tegen virale infecties te vergroten.



CHAPTER 10

APPENDIX

CURRICULUM VITAE
LIST OF PUBLICATIONS
PHD PORTFOLIO
ACKNOWLEDGEMENTS

CURRICULUM VITAE

Rik Anton de Groen was born on April 26th, 1987 in Rochester, Minnesota in the United States of America. In 2005 he graduated, with an honors diploma, from Mayo Senior High School in Rochester and started his study of biology at the College of Biological Sciences, University of Minnesota in Minneapolis/St. Paul. During this time, he also completed 2 undergraduate research fellowships at the Department of Biochemistry of the Mayo Clinic, investigating the role of mutations in mitotic checkpoint proteins in normal and neoplastic growth, supervised by prof. dr. J. van Deursen. After completing his undergraduate degree at the University of Minnesota, he moved to Rotterdam, the Netherlands in 2010 for a half year immunology internship at the Department of Gastroenterology and Hepatology of the Erasmus MC, under the supervision of dr. A. Boonstra. During this internship, he focused on investigating the link between polymorphisms in the interferon-λ gene and resolution of hepatitis C infection. This project would eventually continue under the combined supervision of co-promotor dr. A. Boonstra and promotor prof. dr. H.J. Metselaar, and, in combination with continuation of an ongoing project investigating the role of NK cells in disease state and outcome of viral hepatitis infection, culminate in the work presented in this PhD thesis. In 2016 he started work at Gilead Sciences Netherlands B.V. as a medical scientist in the field of HIV infection, where he is currently employed in the Medical Affairs department in Amsterdam, the Netherlands.

LIST OF PUBLICATIONS

Boeijen LL, Montanari NR, **de Groen RA**, van Oord GW, van der Heide-Mulder M, de Knegt RJ, Boonstra A. Mucosal-associated invariant T (MAIT) cells are more activated in chronic hepatitis B, but not depleted in blood: reversal by antiviral therapy. *J Infect Dis*. 2017 Aug 23. doi: 10.1093/infdis/jix425 [Epub ahead of print]

de Groen RA, Groothuismink ZMA, van Oord G, Kootstra NA, Janssen HLA, Prins M, Schinkel J, Boonstra A. NK cells in self-limited HCV infection exhibit a more extensively differentiated, but not memory-like, repertoire. *J Viral Hepat*. 2017 Apr 17. doi: 10.1111/jvh.12716. [Epub ahead of print]

de Groen RA, Hou J, van Oord GW, Groothuismink ZM, van der Heide M, de Knegt RJ, Boonstra A. NK cell phenotypic and functional shifts coincide with specific clinical phases in the natural history of chronic HBV infection. *Antiviral Res*. 2017 Apr;140:18–24.

de Groen RA, Groothuismink ZM, Liu BS, Boonstra A. IFN-λ is able to augment TLR-mediated activation and subsequent function of primary human B cells. *J Leukoc Biol*. 2015 Oct;98(4):623–30.

de Groen RA, Boltjes A, Hou J, Liu BS, McPhee F, Friborg J, Janssen HL, Boonstra A. IFN-λ-mediated IL-12 production in macrophages induces IFN-γ production in human NK cells. *Eur J Immunol*. 2015 Jan;45(1):250–9.

de Groen RA, Liu BS, Boonstra A. Understanding IFNλ in rheumatoid arthritis. *Arthritis Res Ther*. 2014 Jan 21;16(1):102.

de Groen RA, Mcphee F, Friborg J, Janssen HL, Boonstra A. Endogenous IFNλ in viral hepatitis patients. *J Interferon Cytokine Res*. 2014 Jul;34(7):552–6.

Verstrepen BE, de Groot NG, Groothuismink ZM, Verschoor EJ, **de Groen RA**, Bogers WM, Janssen HL, Mooij P, Bontrop RE, Koopman G, Boonstra A. Evaluation of IL-28B polymorphisms and serum IP-10 in hepatitis C infected chimpanzees. *PLoS One*. 2012;7(10):e46645

PHD PORTFOLIO

Name PhD Student:	Rik Anton de Groen, BSc
Erasmus MC Department:	Gastroenterology and Hepatology
PhD period:	July 2011 - October 2017
Promotor:	Prof. dr. Herold J. Metselaar, MD PhD
Co-promotor:	Dr. André Boonstra, PhD

PHD TRAINING

Courses and Workshops

Workshop Ingenuity Pathway Analysis (IPA), January 2016
Research Management for PhD-Students, December 2012/January 2013
Virology 2012, June 2012
The Basic Introduction Course on SPSS, September 2011
Regular Classes Experimental Gastroenterology, 2011–2015
Essays in Gastroenterology, 2014
Clinical Internship Gastroenterology, January-July, 2012

International and National Conferences

Year	Conference	Presentation	Location
2017	9th International AIDS Society (IAS) Conference on HIV Science	None	Paris, France
2017	Conference on Retroviruses and Opportunistic Infections (CROI)	None	Seattle, USA
2016	Netherlands Conference on HIV Pathogenesis, Epidemiology, Prevention, and Treatment (NCHIV)	None	Amsterdam, The Netherlands
2016	Infectious Disease (ID) Week	None	New Orleans, USA
2016	HIV Drug Therapy	None	Glasgow, Scotland
2016	World AIDS Conference	None	Durban, South Africa
2015	Molecular Biology of Hepatitis B Viruses International Meeting	Poster	Dolce Bad Nauheim, Germany
2015	Nederlandse Vereniging Voor Gastro-Enterologie Voorjaarscongres	Poster	Veldhoven, The Netherlands
2015	19th Molecular Medicine Day	Poster	Rotterdam, The Netherlands
2014	The British Society of Immunology Annual Congress	Poster	Brighton, UK
2014	18th Molecular Medicine Day	None	Rotterdam, The Netherlands

2013	American Association for the Study of Liver Disease (AASLD), The Liver Meeting	Poster	Washington DC, USA
2013	NK2013 – 14th Meeting of the Society for Natural Immunity	Poster	Heidelberg, Germany
2013	Nederlandse Vereniging Voor Gastro-Enterologie Voorjaarscongres	Oral	Veldhoven, The Netherlands
2013	17th Molecular Medicine Day	Poster	Rotterdam, The Netherlands
2013	VIRGO Consortium Scientific Meeting	Oral	Scheveningen, The Netherlands
2013	European Association for the Study of the Liver (EASL) - The International Liver Congress	None	Amsterdam, The Netherlands
2012	European Association for the Study of the Live (EASL) - Immune Mediated Liver Injury	None	Stratford-Upon-Avon, UK
2012	Nederlandse Vereniging Voor Immunologie - APCs revisited: The function of antigen presenting cells in health and disease	None	Lunteren, The Netherlands
2011	Nederlandse Vereniging Voor Immunologie - Time for high T: Features and functions of T-cells in health and disease	None	Lunteren, The Netherlands

FUNDING

Research Funding

Investigator Initiated Study - A Detailed Evaluation of the Biology of the IFN-Lambda Family of Cytokines: Implications for Chronic HCV Infections, 2011–2013 (€103,000.00)
Position: Co-Applicant/Investigator
Source/Collaborator: Bristol-Myers Squibb
Aim: To increase understanding of the basic biology of the IFN-λ family cytokines by an approach that combines clinical and fundamental studies.

Travel Funding

Erasmus Trustfonds Travel Grant, 2014
Erasmus Trustfonds Travel Grant, 2015

TEACHING ACTIVITIES

Organization of Lab-Rotations MSc Students October 2011, 2012, 2013, and 2014

ACKNOWLEDGEMENTS

André, I couldn't have asked for a better mentor and co-promotor during my time as a PhD student. From the moment I moved to Rotterdam for my immunology research internship, you were constantly supportive, even though I may have tested the limits of your patience at times, and were always eager to discuss results or new research ideas and projects. For this I will never be able to fully express my gratitude, but I hope this thesis is some small measure. I look forward to seeing where your research takes you next, and hopefully we can sit down together again soon to brainstorm or maybe just discuss the difference between Dutch and American politics and culture.

Dear **Herold**, thank you for the your guidance and support in completing my thesis and preparing for my thesis defense. You were always able to help me look at the big picture and see what needed to be done to complete this seemingly monumental task. For this I will always be grateful and wish you all the best in the future.

Harry, I would have never come to Rotterdam if it wasn't for you, and now I couldn't imagine my life anywhere else. It truly has become my new home. Thank you for giving me a chance and the support over the years, and I wish you all the best in your new home in Toronto.

Maikel, thank you for your valuable input over the years during seminars, and for guidance and leadership in the lab.

To **Prof.dr. Baan, Prof dr. Boucher, Dr. Arends, Prof.dr de Man, Dr. Debets, Dr. Claassen, and Dr. Schinkel**; Thank you all very much for being a part of my thesis evaluating and defense committee, it truly is an honor to be able to discuss my research with each and every one of you.

Marion, I know it was often from a distance, but thank you so much for your support and helping make this PhD process as smooth as possible (even through all the long bureaucratic processes). My deepest gratitude for this!

To my paranimfen;
Wesley, I'm probably not the first to say this, and definitely won't be the last, but I can't think of anyone that exemplifies the adage "still water run deep" more than you. You've become one of my best friends over the past years, and even went out of your way to adopt me into "Team Whisky". I can never really thank you enough for this. It was an honor to stand next to you during your defense, to be a master of ceremonies at your and Cindy's wedding this September (I still think

you two are crazy for asking me ;P), and now to have you standing by my side on my big day. I wish you, **Cindy**, and **Jeremy** all the best in Maastricht the coming years (hopefully you'll be moving back to Rotterdam at some point!), and keep the spare bedroom ready because I'll be coming by often to visit and work on my Limburg's accent.

Anthonie, who else can say that they learned how to FACS and culture/stimulate cells from that same person that taught them how to open a beer with their shoe? You're truly the master of all in the lab and the foundation of the viral hepatitis team. Thank you so much for teaching me the basics (and advanced techniques) of immunology in the lab, for all the help with my projects over the years, and of course the late nights singing Disney karaoke and riding the nachtnet back from Utrecht. We've worked together since the day I moved to Rotterdam and started at the Erasmus MC, and that's why I couldn't think of anyone more fitting to stand next to me on my last day. Keep in touch, and let me know if you ever catch the lab vampire that bit Paula or the Kootwijk Killer ;)

To the viral hepatitis groups;

To **Gertine, Kim, and Marieke**; Thank you ladies for all of the help and endless hours of culturing, stimulating, harvesting, FACSing, and cell-sorting (to name just a few…). I don't know what I would have done without you three, and I'm sure the same goes for the entire viral hepatitis group ;)

Jun, Bisheng, and Arjan; It has been a pleasure collaborating with each of the past years on the macrophage, B cell, and NK cell studies. I wish you all the best in the future and keep in touch!

Rob, Thomas, and Betina; thank you for your clinical and statistical insights and support over the years, this definitely helped with my overall development as a PhD student.

Andrea, thank you for all of your input and advice during viral hepatitis meetings and seminar over the years.

Paula, Aniek, Nadine, Sonja, Monique, Dowty, Boris, and Noé, working together with all of you in the lab and celebrating during our office parties has been an absolute blast the past years. Please keep this atmosphere alive for future PhD students.

To the Man Cave, BOTM, Cadzand-goers, campers, Oktoberfesters, and many other friends;

Elmer, my fellow caveman. You waddled (sorry…) in and out of the office every day with a smile on your face and that was contagious. Your kind-heartedness and natural magnetism always seems to draw people toward you and for good reason. You've become a great friend over the past years, and I couldn't imagine my PhD time without you and Wesley in the "Man Cave". The occasional wiener melange, put-put, blind beer-tastings (preferably alcohol-free), and whisky on the beach to name just a few of the memories we've made over the years. Best of luck with finishing your MDL specialization, although I know you don't need it, and hopefully this year we can celebrate "3 Oktober" on the 10th of October instead!

Evelyn, I've said it before but I'll say it again, I can't think of anyone more deserving of their Dr. title than you. Your relentless determination as well as genuine thoughtfulness and kindness made you both a great colleague and an even better friend. Thank you for bringing some female perspective and balance to the male dominated "Man Cave" shenanigans, I'm sure it wasn't easy to tolerate at times. I wish you all the best with your new career at J&J, and I'm sure you'll be successful wherever life takes you (you've always got a promising career as a clown to fall back on ;P).See you around Dr. van der Aa.

Michelle, we started in the viral hepatitis group together and I can't say how thankful I am for that. It was always great to have someone, that was also relatively new, to bounce ideas off and work together in the lab. You've been in London the past couple years, and are finally moving back to Rotterdam (which Team BOTM is very excited about!), so I hope we can finally all grab a drink together soon. Best of luck with starting your journey into MDL specialization, and don't be a stranger!

Vincent, how am I supposed to summarize someone as unique as Vincent Janmaat in just a few sentences? Maybe I'll write a book about you someday. Until then, thank you for the many, many fantastic memories over the past years. You're a great friend and gym/running-buddy, and I was beyond ecstatic to hear you are staying in Rotterdam for you MDL specialization. When is pole-camping expedition #2? I want to catch a real Loch Ness monster next time.

Lauke, if it would take me an entire book to thoroughly address Vincent Janmaat, it would take an entire encyclopedia collection to comprehensively cover Lauke Boeijin. I swear you two are two peas in a pod, or should I say two goldfish in a bowl (who's Rik and who's Elmer???)? From Le Lac De Zombies and Oktoberfest in Dolce Bad Nauheim, to BBQ and beer pong belts in Egmond aan Zee, the past two years have been a riot. Unfortunately you've moved to Utrecht now, but you've always got a couch to crash on at the Oude Binnenweg.

Martijn, chef BBQ maestro! You've introduced us to so many great craft beers over the years, and I'm therefore very happy we could repay the favor by introducing you to Jupiler ;) Best of luck in Utrecht with your new job, and of course with your upcoming thesis defense!

Jasmijn, even despite your frequent absence due to scheduling conflicts (weekends in Paris, every festival known to man, Thursday night Ikea trips, etc.) we were still able to hang out every blue moon ;) You're directness and sense of humor are refreshing, and try to keep some weekends open this year, so we can all hang out with the Roffa crew.

Xiaolei, my mentor in all things Chinese. Your Eastern wisdom (and general absentmindedness) made for an very diverse and entertaining "Man Cave". I'm very glad you decided to come back to the Netherlands, and it's great to have a surf and Simonis fish buddy in Den Haag. Surfs up, see you soon, and best of luck with your journey into patent law ;)

Guoying and Yingying, the other 2 ambassadors from China! It's been a pleasure to work and learn about Chinese culture from you both, and I have incredible respect for your interest and curiosity in Dutch (and even American!) culture. Thanks to you both, hot pot has become a staple dish for me at home, and I hope to continue to improve my Chinese cooking skills the coming years! We'll stay in touch, and best of luck to you both with the last stretch of your PhD time.

Michiel and Floris, I believe the lab "party committee" has been left in capable hands. It's up to you two to keep the Oktoberfest and camping traditions alive, and I have nothing but the utmost confidence you will do so (no pressure) ;)!

Jasper and Werner, it's been a few years since you've left the lab, but thank you both for being so welcoming and inviting when I first moved to the Netherlands to work in the lab. Hopefully we'll see each other again soon!

Reneé, Eelke, Emmeloes, the first female residents of the "Man Cave". Thank for the great times and many laughs, I wish you all the best in your diverse paths in the future.

Leonie and Gwenny, the Bert and Ernie of the lab ;) (don't get too mad, it's meant to be endearing)! We've shared many a beer and glass of wine, and it usually only ended when the last bottle was empty. Always a tragedy! Thank you both for the friendship and support with my PhD over the years, and especially for the extra effort you've put in with helping me complete the last items for my thesis defense Leonie. See you both at the next borrel ;)!

Arjan "I Could Eat" Rauwers, stay hungry! What can I say roomie, we've had some great times and there are many more ahead. Road tripping through America (even in a red Ford Mustang…) was amazing, and I have high expectations for Lowlands 2018. Thanks for helping me tap into my Dutch side, and I hope I've been able to pass on some of my American wisdom to you… Trump 2016!

Dak Duifjes, thank you for the great memories during the Friday borrels, Rotjong games, and of course the legendary department ski trips ;P. I wish you all the best in the future!

To **Raymond, Jan, Buddy, Frances, Ester, Martine, Hanneke, Auke, Greta, Jaap, Alexander, Rogier, Patrick, Shanta, Kostas, Jesse, Pieter, Luc, Henk, Petra, Monique, Suomi, Elvira, Wendy, Susanne, Waqar, Sohrab, Ron, Marcel, Huga, Pauline, Abdullah, Wenshi, Yuebang, Lei, Xingying, Juan, Changbo, Wanlu, Wen, Wenhui, Kan, Hakim, Rajesh**, and countless lab-mates I'm probably forgetting, thank all you so very much for making it an enjoyable 5 years, both in and out of the lab, with endless banter in the coffee/lunchroom and unforgettable lab/department events and parties.

Team Futsal and Whisky thanks for the many great times and adopting me for the yearly excursions throughout Europe. I'm looking forward to another epic weekend in Glasgow in November! I would also like to thank all the **healthy controls and patients**, that so generously donated blood and tissue to make this research possible. Without them, this thesis would not exist.

Nick, Brad, Eric, Dean, John, and Mark, I've known most of you longer than I can remember, and never really understood how lucky I was to have you all as friends until after I left the USA. I look back fondly on the many memories we've made over the past ≈ 30 years, and look forward to the ones yet to come. See you boys on the other side of the pond!

Bhrunil, Bryce, Lukas, Zac, **Clay, Mohammed**, **and my other university friends**, thanks for the great memories and the time we spent together during our first years of adulthood. You've all had a profound impact on me, I miss you all, and we need another reunion soon!

Lieve familie de Groen en Janssen, I could write second entire dissertation on the things I need to be thankful for when it comes to my family. I'd prefer to do it in person, and hopefully one day repay the favor. I would like to say a special thank you to **Harry, Godelieve, and Lodewijk**, who helped really make Rotterdam my new home. I can never thank you three enough for this.

Opa, I've saved a spot for you front and center. It took a few generations, but someone will finally defend their thesis. I hope I've made you as proud as I am to have you here today.

Max, Ryah, Madeleine, and Anton crazy Uncle Rik is beyond ecstatic to have all 4 of your sitting here today. I look forward to watching Madeleine and Anton grow up, and giving them both the noisiest of toys every birthday (what are your thoughts on a drum set?).

Rob, I can't tell you how much I've missed you over the past 6 years. It means the world to me that you are hear today, and that you can share in celebrating this occasion with me. Talk soon little brother.

Mom and Dad, this book is as much a product of you both as it is of me. Thank you for the years of unwavering support, although sometimes overbearing, I always knew it came from the right place. I hope I've made you proud today.

Timshel.