The effect of interferons and viral proteins on antigen-presenting cells in chronic hepatitis B

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The effect of interferons and viral proteins on antigen-presenting cells in chronic hepatitis B

Het effect van interferonen en virale eiwitten op antigeenpresenterende cellen in chronische hepatitis B

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Abbreviations

APC	Antigen-presenting cells
cccDNA	Covalently closed circular DNA
DC	Dendritic cells
GM-CSF	Granulocyte macrophage colony-stimulating factor
GZMB	Granzyme B
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B early or pre-core antigen
HBpAg	Hepatitis B polymerase
HBsAg	Hepatitis B surface or envelope antigen
HBV	Hepatitis B virus
HBxAg	Hepatitis B x antigen
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HSPG	Heparan sulfate proteoglycan
IFN	Interferon
IFNλR1	Interferon-λ receptor 1
IFNAR	Interferon-α/β receptor
IL(-6)	Interleukin(-6)
IMPDH	Inosine-5′-monophosphate dehydrogenase
ISG	Interferon-stimulated gene
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response elements
KC	Kupffer cells
LBP	LPS-binding protein
LCMV	Lymphocytic choriomeningitis virus
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MCMV	Murine cytomegalovirus
mDC	Myeloid DC
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MHV	Mouse hepatitis virus
MoMΦ	Monocyte-derived macrophages
NA	Nucleos(t)ide analogues
NK cells	Natural killer cells
NLR	NOD-like receptors
NPC	Non-parenchymal cells
NTCP	Na^+-taurocholate cotransporting polypeptide
ORF	Open-reading frame
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid DC
Peginterferon	Pegylated interferon
pgRNA	Pregenome RNA
pHBsAg	HBV patient's plasma-derived HBsAg
PKR	Protein kinase R
PRR	Pattern recognition receptor
rHBsAg	Recombinant HBsAg
RLR	RIG-like receptor
ROS	Reactive oxygen species
sCD14	Soluble CD14
SR	Scavenger receptor
SVR	Sustained viral response
T_H1/2/17	T helper cell type 1, 2 or 17
TLR	Toll-like receptor
TNF	Tumor necrosis factor

General Introduction

Chapter 1

Antigen-presenting cells and interferons

Antigen-presenting cells in antiviral immunity

When the body is exposed to a virus the innate immune system forms the so-called first line of defense against invading pathogens. Innate immunity encompasses physical and chemical barriers like epithelial surfaces; blood proteins like the complement system; and immune cells including natural killer (NK) cells, and phagocytes like monocytes, macrophages and dendritic cells (DC) [1]. Monocytes, macrophages and DC (Figure 1) sample their environments, binding and taking up viral pathogens to be destroyed or further processed, thereby limiting the spread of the pathogen. Furthermore, these cells are able to recognize viral pathogens by means of their pattern recognition receptors, like Toll-like receptors (TLR) and c-type lectins. Virus recognition will lead to the activation of phagocytes that will induce them to produce chemokines and cytokines, small proteins that not only attract and activate other immune cells, but also have direct antiviral capacities like interferon alpha (IFNα), interleukin (IL)-6, and TNF. Other immune cells will, upon activation, also secrete cytokines like IFNγ, reciprocally further activating innate cells [2, 3]. Additionally, monocytes, macrophages and DC are professional antigen-presenting cells (APC). Professional APC are capable of presenting pathogen-derived antigens to T cells. DC have the unique property to take up antigen from the periphery, migrate to a local lymph node and prime naïve T cells into T effector cells via an interaction between the T-cell receptors and DC-expressed MHC molecules (1st signal), via co-stimulatory signals (2nd signal) and via cytokines (3rd signal). DC and other APC can induce further T effector cell, but also B cell, activation and differentiation at the site of inflammation, ultimately leading to the induction of virus-specific T and B cell responses [1].

Dendritic cells

Among professional APC, particularly efficient antigen-presenting cells are DC – cells that are investigated in chapter 3. As mentioned above, in contrast to macrophages they are able to migrate from the periphery to lymph nodes to activate naive T cells. In peripheral blood, within the HLA-DR$^+$ lineage-negative fraction, human DC can be divided into three main subsets: plasmacytoid DC (pDC) and two types of myeloid DC (mDC); BDCA-1/CD1c$^+$ and BDCA-3/CD141$^+$ mDC. BDCA-3$^+$ mDC only make up 5-10% of the total peripheral blood DC population, while the remainder is divided into equal parts pDC and BDCA-1$^+$ mDC. While both mDC subsets highly express CD11c, pDC are defined by the expression of CD123, BDCA-2 and BDCA-4 and expression of CD11c is low or even absent [4, 5]. mDC are especially well known for their efficient induction of T-cell proliferation in general, and IL-12-induced antiviral T_H1 responses in particular [6, 7]. Furthermore, BDCA-3 mDC are able to produce IFNλ and are especially well-known for their cross-presenting capacity [6, 8, 9]. pDC, on the other hand, are best known for their high level type I IFN production, and to a lesser extent for their type III IFN production, in response to TLR7 and TLR9 triggering, while their T-cell-inductive capacities are poor [10-12]. Single-stranded RNA and CpG motifs found in DNA that trigger TLR7 and TLR9, respectively, are commonly found in viruses or bacteria. The induction of IFNα, but also of other

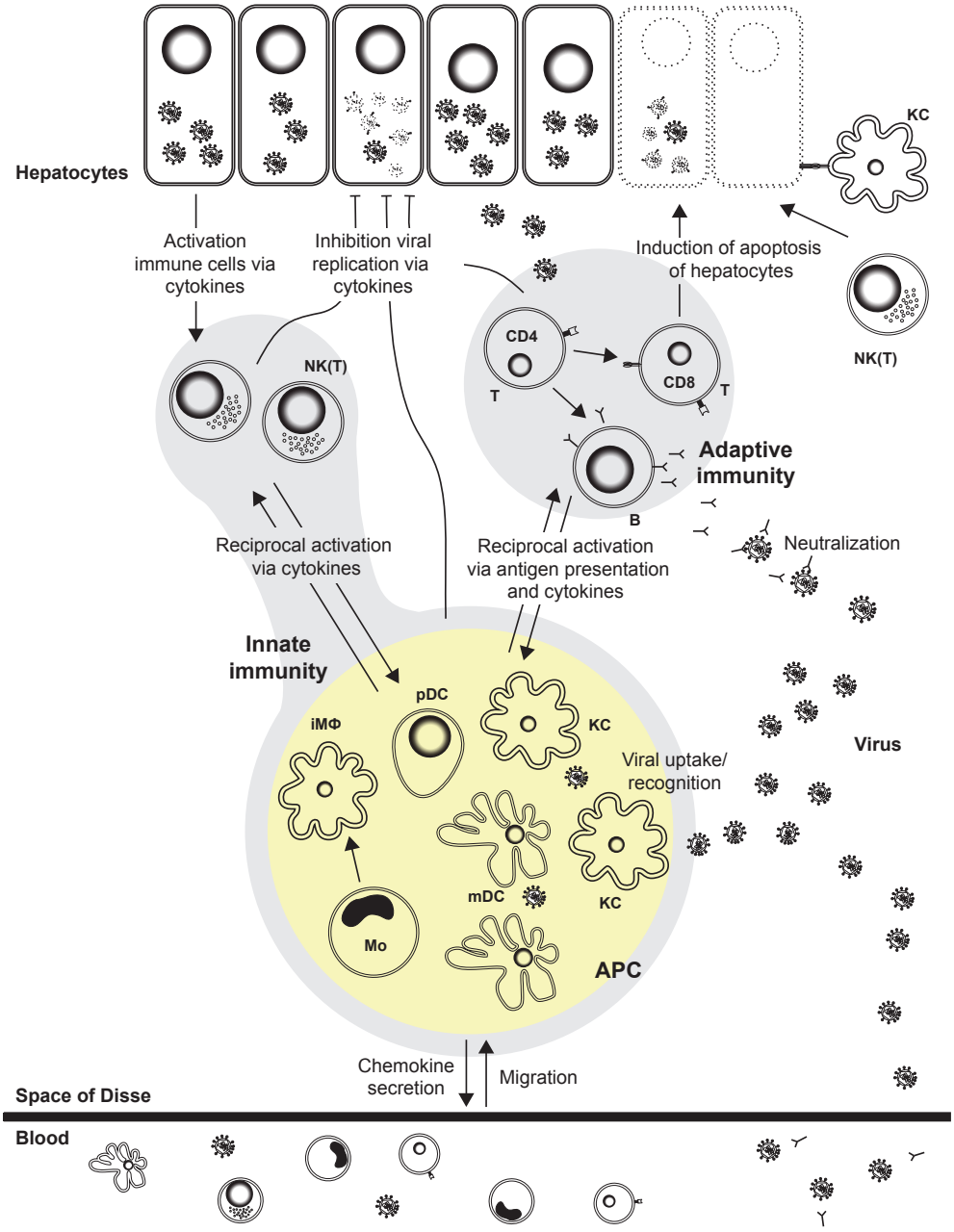

Figure 1. The role of APC in antiviral immunity during virus infection. Exposure of APC to a virus will lead to direct activation of APC that, together with infected hepatocytes, release cytokines and chemokines, which are responsible for the attraction of other leukocytes. Activation of infiltrating immune cells leads to further production of cytokines that indirectly activate APC. The secreted cytokines may inhibit viral replication. Additionally, activated KC, NK cells and $CD8^+$ T cells are able to induce apoptosis of infected hepatocytes. Activated B cells produce virus-specific neutralizing antibodies. * HBsAg induces IL-23 *in vitro* in monocyte-derived DC and monocyte-derived macrophages; possibly *in vivo* in liver macrophages and DC.

cytokines like TNF and IL-6, has strong direct antiviral effects in addition to affecting surrounding immune and non-immune cells [13]. By presenting antigens and producing pro-inflammatory cytokines but also IL-10, DC play a central role bridging the innate and adaptive immunity, thereby not only initiating but also regulating the adaptive immune response in a quantitative and qualitative way [14-17].

Monocytes

Human blood monocytes are far more abundant than blood DC, representing about 10% of total peripheral blood mononuclear cells (PBMC), and like DC they can be subdivided into different subsets based on phenotype and cytokine production. The main subset in peripheral blood is $CD14^{hi}$ $CD16^{-}$ monocytes, making up 80-90% of blood monocytes. These often called “classical monocytes” further express high levels of CCR2, low levels of CX3CR1, and rather produce IL-10 than TNF and IL-1 upon LPS triggering. A second subset is made up of $CD16^{+}$ monocytes, that can be further subdivided into a $CD14^{hi}$ $CD16^{+}$ subset and a $CD14^{dim}$ $CD16^{+}$ subset, that represent the main producers of inflammatory cytokines like TNF [18, 19].

The traditional view that monocytes are simply precursors of macrophages and DC has slowly been extended by the observation that also monocytes themselves can play an important role as an innate immune cell in the first-line defense against pathogens, driving inflammation complementary to DC [20]. However, despite being called a professional APC, expressing high levels of MHC class II and being able to retain and present antigen-derived peptides, monocytes are poor antigen presenters [18, 21]. Alternative functions like cytokine production may however contribute to supporting antiviral immunity.

Macrophages

Macrophages can roughly be divided into two types: tissue-resident and infiltrating macrophages. Tissue resident macrophages are, as opposed to DC and monocytes, long-lived non-migratory cells that are specialized in taking up and processing dead cells and debris. They possess high proteolytic activity and are often poor antigen presenters. They play an essential role in maintaining tissue homeostasis by clearing cell debris and promote resolution of inflammation and wound healing [22]. However, tissue resident macrophages can also promote antiviral immunity and inflammation by the production of chemokines like CCL2, CXCL1 and MIF, and cytokines like IL-6 and TNF, attracting and activating other immune cells, as discussed for Kupffer cells (KC) – the macrophages of the liver – in chapter 2 [13].

The second type of macrophages, the infiltrating macrophages, derive from monocytes that are recruited to tissues in inflammatory conditions. In the human setting, there is still no marker to distinguish infiltrating macrophages from resident macrophages which makes it hard to study subpopulations. Infiltrating macrophages can be divided into three main populations, with a spectrum of macrophage subpopulations in between, based on function, displaying either a pro-inflammatory profile (originally coined “classically activated” or “M1” macrophages), a regulatory profile, or a wound-healing profile (both originally grouped under the term “alternatively activated” or

"M2" macrophages) depending on the tissue context and environmental stimuli [23, 24]. In this respect, the monocyte-derived macrophages that were used in chapter 4 demonstrate an M1-like activity, while the monocyte-derived macrophages from chapter 6 show an M2-like activity.

Interferons

Key molecules in the induction/regulation of an antiviral response are the IFN. They not only have direct antiviral effect, inducing IFN response genes in infected and non-infected cells, but also execute their antiviral functions indirectly by modulating immune cells [25]. IFN are subdivided into three groups: type I IFN, the largest group encompassing IFNα, IFNβ and other less-defined members; type II IFN with only a single member, IFNγ; and type III IFN, comprising IFNλ1 (IL-29), IFNλ2 (IL-28A) and IFNλ3 (IL-28B) [2, 25, 26]. Although IFNγ is an important cytokine, and we show its production in several experiments in the various chapters, our focus is on IFNα and IFNλ in chapters 3 and 4, respectively.

Of type I IFN, IFNα and IFNβ are induced in response to viral infection. Almost all nucleated cells respond to viral infection by producing type I IFN, but especially pDC are very potent type I IFN producers [27]. There are 14 subtypes of IFNα, and although exact expression profiles and specific roles for IFN subtypes need to be elucidated it seems that IFN types are expressed in a cell- and ligand-specific fashion and have effects that are functionally specialized and subtly unique [28-30]. All type I IFN bind to the same receptor known as the IFNAR, a heterodimer of IFNAR1 and IFNAR2; a receptor ubiquitously expressed. Binding of the receptor leads to JAK1/TYK1-regulated phosphorylation of IFNAR1, STAT1 and STAT2, which leads to dimerization of STAT1 and STAT2. Consecutive transport of this dimer to the nucleus enables association with IRF9, forming the IFN-stimulated gene factor 3 (ISGF3) complex. This heterotrimer binds to IFN-stimulated response elements (ISRE), present in the promotor regions of most ISG, to induce transcription of hundreds of ISG [31] for which the function in antiviral immunity often remains to be elucidated. Some well-known ISG that have antiviral effects are 2',5'-oligoadenylate synthetase (OAS) that modifies and degrades viral RNA; protein kinase R (PKR) that limits cellular translation; myxovirus resistance (Mx), a guanosine triphosphatase that alters cellular vesicle trafficking. Ultimately, type I IFN have been shown to be able to inhibit replication of various viruses, including HBV [32]. Besides inducing ISG with direct antiviral effect, IFNα also has indirect antiviral effects like stimulating the proliferation and accumulation of NK cells and modulating the activation of $CD8^+$ T cells leading to increased IFNγ [25, 32].

Type III IFN, like type I IFN, are induced following viral infection. Both non-immune cells among which are hepatocytes and immune cells can produce type III IFN [9, 12, 33, 34]. Especially pDC and BDCA-3^+ mDC are potent IFNλ producers [9, 12]. All three IFNλ members bind to the IFNλ receptor, a heterodimer of IL-10R2 and IFNλR1. In contrast to IFNAR expression, the IFNλ receptor displays a more restricted expression, mostly caused by the limited expression pattern of the IFNλR1 subunit. Despite this difference in receptors, and although IFNλ shows little homology with IFNα, binding of IFNλ to its receptor induces a signalling similar to that induced by IFNα/β binding to its receptor, involving JAK1/TYK1, STAT1, STAT2 and IRF9, the latter three again forming ISGF3. Consequently, type III IFN signalling induces a

gene expression profile in human liver cell lines and other cell lines nearly identical to that induced by type I IFN [35]. Additionally, IFNλ is able to inhibit a wide variety of viruses including HBV in murine liver cell lines, most likely caused by the same antiviral mechanism utilized by type I IFN [35, 36].

However important the role of IFN may be in antiviral immunity, in contrast to HCV and other viruses, HBV does not seem to induce any ISG in the livers of chimpanzees during the early stages of infection [37], nor *in vitro* in primary human hepatocytes [38]. And although it is difficult to assess such early events in acute HBV infection in humans it was recently shown that type I IFN responses are also absent in acute HBV patients [39]. Since the IFN response induced by IFNλ largely overlaps with that induced by IFNα/β, it seems unlikely that IFNα/β or IFNλ play a big role in antiviral immunity in HBV infection. However, since IFN are able to inhibit HBV infection, their use as antiviral agents in HBV therapy provides them a role in antiviral immunity against HBV after all.

Hepatitis B virus

Hepatitis B virus (HBV) is a hepatotropic virus causing both acute and chronic disease. Although the majority of infected adults spontaneously clear the virus, a small part of patients is unable to do so, developing a chronic form of hepatitis. Currently over 240 million people are chronically infected by HBV, which can lead to progressive liver damage that increases the patient's risk of developing liver cirrhosis, liver failure and liver cancer. These consequences of HBV infection pose a serious global health problem, culminating in the death of about 600,000 people every year [40, 41].

HBV course

HBV is transmitted via perinatal, percutaneous and sexual exposure [42]. Infection will lead to an initial acute hepatitis during which symptomatic patients are presented with jaundice, dark urine, extreme fatigue, nausea, vomiting and/or abdominal pain, but in most patients the infection is asymptomatic [41, 43, 44], especially in children [45]. After the initial phase, the risk of developing chronic HBV infection depends on age, infection route and dose, ranging from 90% in neonates to less than 10% in adults. Accordingly, most adults spontaneously clear the virus, and therewith acquire lifelong protective immunity [40]. If spontaneous clearance does not take place and chronic infection ensues, uncontrolled infection may lead to chronic hepatitis, liver fibrosis and cirrhosis, which may result in hepatic failure, hepatocellular carcinoma (HCC) and ultimately death [40].

Clearance of the infection is likely due to the combination of an efficient innate immune response and a strong, sustained, and multi-specific T cell-mediated immune response [2, 46]. Indeed, in order to control HBV, sustained, vigorous and multi-epitope-specific $CD4^+$, $CD8^+$ T cell responses are essential [46], and following clearance of the virus in the acute phase, long-term memory T cell responses are detected [47, 48]. Also B cell antibody production is important, neutralizing free viral particles and preventing (re)infection [49]. Moreover, in chronic HBV, virus-specific

T and B cell responses are weak, transient and targeting only few epitopes [46]. It is generally believed that this weak adaptive immunity results, in part, from an ineffective hepatic innate immune response in which APC play a prominent role [2, 13, 17, 46].

HBV virology

HBV, member of the family Hepadnaviridae, is a DNA virus containing a genome of 3.2 kb partially double-stranded DNA. Its four open reading frames encode for the five HBV proteins: polymerase (HBpAg), envelope or surface (HBsAg), core (HBcAg), early or pre-core (HBeAg), and X (HBxAg) protein. In infected cells, core particles, made up of HBcAg capsids containing the genomic DNA and viral polymerase, are transported into the nucleus where the genomic DNA forms covalently closed circular DNA (cccDNA). This cccDNA serves as a template for viral mRNA transcription by host RNA polymerase II. Viral RNA subsequently is transported to the cytoplasm where it is translated into viral protein, except for the 3.5 kb pre-genomic RNA, which is encapsulated by HBcAg for replication purposes. Viral polymerase reverse transcribes pre-genomic RNA into the negative genomic DNA strand, which is followed by positive DNA strand synthesis. Ultimately, genomic DNA- and polymerase-containing core particles bud off into the endoplasmatic reticulum to be enveloped by HBsAg, forming a complete viral or Dane particle (Figure 2) that is secreted from the cell. Besides serving as envelope, HBsAg is secreted as empty spheres and filaments. HBV particles, HBsAg and secreted HBeAg can be detected in HBV patients' serum, where HBsAg can outnumber HBV particles (measured as HBV DNA) >10,000 to 1 [40, 50].

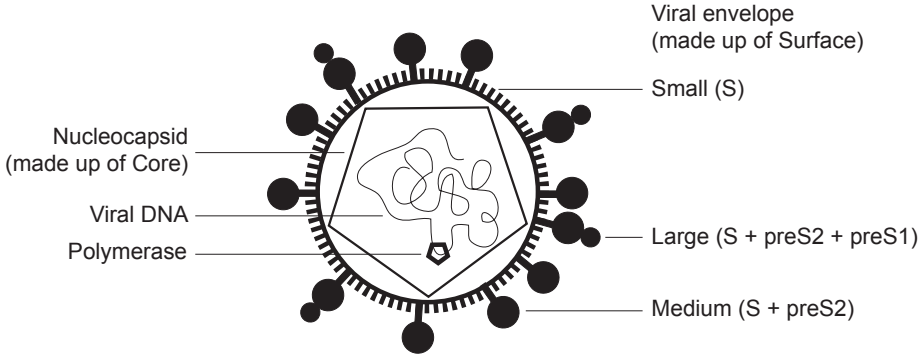

Figure 2. Schematic representation of HBV virion. The viral DNA and the polymerase are packed within the nucleocapsid, which is made up of the core protein (HBcAg). Surrounding this capsid is the viral envelope, made up of the surface protein (HBsAg) in three different sizes of which the small HBsAg is the most abundant.

Viral proteins

Of the five viral proteins, HBeAg and HBsAg are assessed in chapters 5 and 6. Being a truncated form of HBcAg, HBeAg, after transcription of the HBV core gene, is N- and C-terminally processed and secreted as a 159 amino acids-long protein. The function of HBeAg remains unclear: it is not a structural protein and dispensable

for viral replication. However, since HBeAg is conserved in all hepadnaviruses, it is likely that this protein has a yet undefined function [51]. HBeAg seems to have some modulatory capabilities (Figure 3). Pretreatment of hepatocytes or liver non-parenchymal cells, including KC, with HBeAg almost completely abrogated TLR-induced antiviral activity like IFNβ production and ISG induction [52]. Accordingly, incubating human monocytes with HBeAg inhibited TLR2-induced phosphorylation of p38 MAPK, and subsequent production of TNF [53, 54]. *In vivo*, TLR2 expression by KC and peripheral blood monocytes in HBeAg-positive chronic HBV-infected individuals was lower than that in HBeAg-negative patients and controls. Moreover, TLR2 ligation induced less IL-6 and TNF in those HBeAg-positive patients [54]. These alterations may be related to the inhibitory effect of HBeAg on TLR2 signaling demonstrated *in vitro*. HBeAg also slightly inhibits TLR9-mediated cytokine production by pDC [55]. Interestingly, exposure to HBeAg *in utero* establishes T cell tolerance to HBeAg in a murine transgenic model perhaps representing a viral strategy to predispose neonates born to HBV-infected mothers to persistent infection [56].

HBsAg is a heavily glycosylated lipoprotein that is produced in three different sizes all transcribed from the same ORF. Which size is produced upon translation depends on the start codon used. The small (S) HBsAg is made up of the 226 amino acids (aa)-long common S domain, the middle (M) HBsAg is the S domain plus an additional preS2 domain (55 aa), while the large (L) HBsAg contains also a preS1 domain (108-119 aa) besides S+preS2. The S form is the major constituent of both the viral particle envelope and subviral particles. The M and L forms are mostly found as parts of the viral envelope [57-59]. Besides its major function as viral envelope, HBsAg also seems to play a part in the modulation of the immune system, although some effects seem to be complementary (Figure 3). Like with HBeAg, pretreatment of hepatocytes or liver non-parenchymal cells with HBsAg nearly nullified TLR-induced antiviral activity [52]. Also, incubating human monocytes with HBsAg inhibited TLR2- and TLR4-induced production of IL-1β, IL-12 and TNF, although this could only be shown for recombinant HBsAg, not patient-derived HBsAg [59, 60]. HBsAg was also shown to inhibit TLR4-induced IL-12 and IL-18 production in the monocytic cell line THP-1 [61], while in pDC TLR9-induced, but not TLR7-induced, cytokine production and co-stimulatory molecule expression was reduced [55]. In mDC, maturation was inhibited, but – in contrast to other cell types mentioned above – not cytokine production [62]. The inhibition of both monocyte cytokine production and DC maturation may impair subsequent activation of the adaptive immunity and NK cells and its production of IFNγ. However, in contrast to HBeAg, HBsAg as well as HBV not only seem to inhibit immune cell function, but also to activate immune cells, possibly initiating the antiviral immune response to HBV. While pDC have been shown not to be activated by HBV and its components [55], and for mDC this needs to be further examined, liver macrophages ($CD68^+$) and a $CD11c^+$ intrahepatic population produce IL-23 during HBV infection [63]. HBsAg turns out to be able to induce this IL-23 production, but also IL-12 production, in monocyte-derived DC [63, 64]. Furthermore, Hösel *et al.* showed that non-parenchymal cells, presumably KC, react to HBV by secreting pro-inflammatory cytokines IL-1β, IL-6, TNF and CXCL8 [38]. Finally, HBsAg also induced IL-23 expression in monocyte-derived macrophages [63], while monocytes produced TNF and IL-10 upon HBsAg exposure [65].

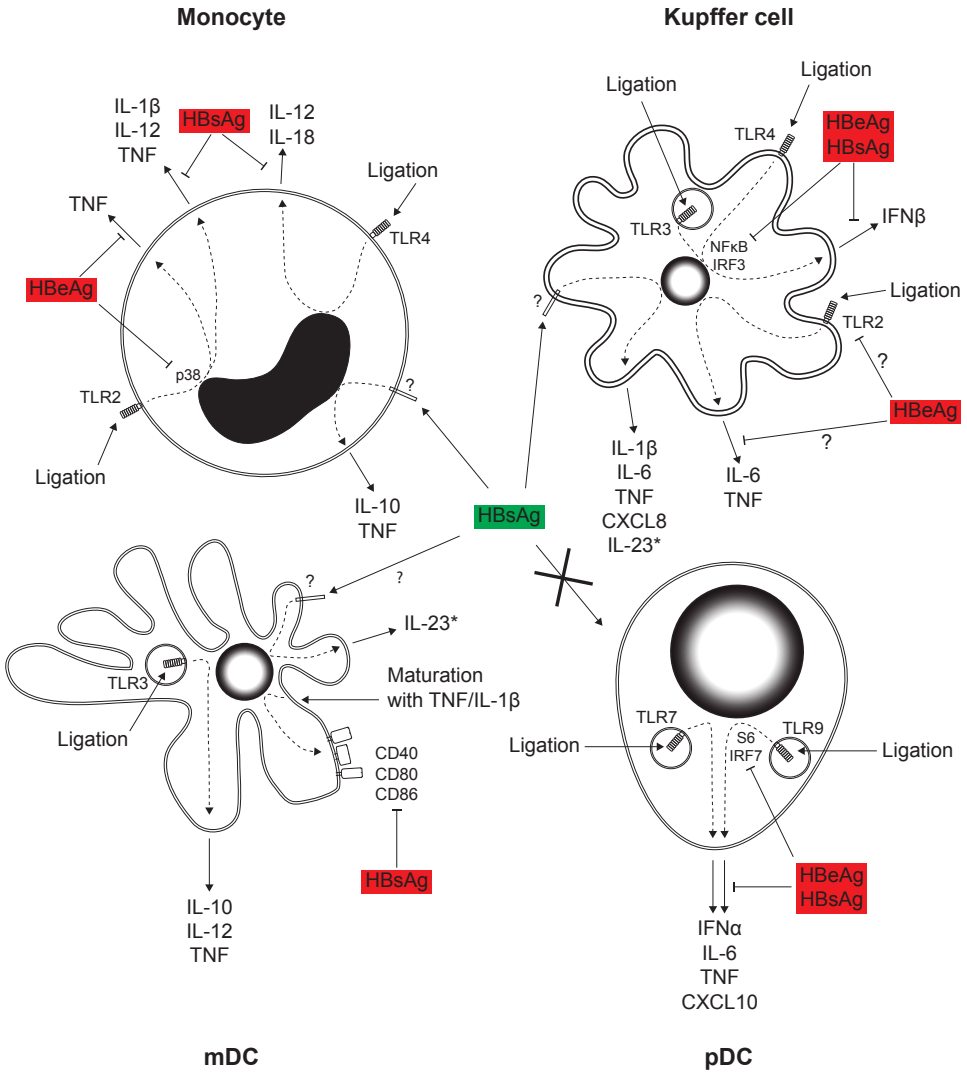

Figure 3. Effects of HBeAg and HBsAg on monocytes and DC subsets. Depicted are the inhibitory effects (⊥) that HBeAg and HBsAg (in red boxes) have on TLR and co-stimulatory marker expression, signaling cascade molecules expression and cytokine/chemokine production by APC. Also depicted are the activatory effects (↓) that HBsAg (in green box) has on cytokine and chemokine (green) expression by APC.

Treatment

First-line treatment of chronic HBV infection comprises two different types of drugs: IFNα, mostly in a pegylated form (PEG-IFNα or peginterferon), and nucleos(t)ide analogues (NA). The advantages of IFNα treatment are its finite duration, the lack of induction of resistant HBV strains [66] and it was thought to have a higher chance of reaching HBsAg loss than NA treatment. Furthermore, unlike NA, peginterferon

can lead to durable off-treatment HBeAg seroconversions after a finite duration of therapy [66-69]. Although no real head-to-head comparisons of the current NA and peginterferon have been performed, current studies show increases in HBsAg loss rates similar to peginterferon treatment after prolonged use of NA [70-72]. On the downside, the efficacy of IFNα to obtain viral suppression and HBeAg seroconversion is merely moderate and its use is accompanied by more severe side-effects than NA treatment. NA treatment on the other hand offers a very potent antiviral effect, can be administered orally and is generally well-tolerated [66]. A disadvantage is the indefinite duration. With the emergence of newer NA, including tenofovir and entecavir, the risk of resistance is negligible in treatment-naïve patients [70]. Depending on the clinical situation, either a finite peginterferon treatment duration with sustained off-treatment responses or continuous well-tolerated and powerful antiviral suppression with NA can be chosen [73]. For the latter choice, the most potent drugs with the optimal resistance profile should be used, i.e. tenofovir and entecavir. Although several clinical trials are examining combinations of IFNα with NA, or combinations of NA, none have up until now shown any advantage above monotherapy with either peginterferon or NA [66]. Some immunological effects of combination of IFNα and an NA, in this case ribavirin, are described in chapter 3.

Since treatment for chronic HBV results in complete viral clearance defined as HBsAg loss in only a minority of the treated patients, the search for more potent therapeutic options is ever ongoing. One drug that has received some attention is IFNλ; a type of IFN that we focus on in chapter 4. Like IFNα, IFNλ inhibits replication of HBV in HBV-Met cells, a differentiated murine hepatocyte cell line [74]. As the receptor for IFNλ is restricted to certain cells, in contrast to the ubiquitous expression of the receptor for IFNα [75-79], it is expected that the effects of IFNλ are much more focused without a lot of the side-effects associated with IFNα treatment.

Antigen-presenting cells in HBV infection

As described above for antiviral immunity in general, in case of HBV infection APC are key players in the initiation and regulation of antiviral immune responses, taking up viral antigen leading to virus recognition, production of pro-inflammatory cytokines and presentation of viral antigens, inducing adaptive immunity. This ensuing combined cellular and humoral immunity ultimately allow control of HBV infection [13, 17] as depicted in Figure 1.

When immune control over HBV is lacking, both innate and adaptive immune responses are weak. Decreases in numbers of DC have been described in the peripheral blood of chronic HBV patients, inversely proportional to ALT levels that reflect liver damage [80, 81]. In contrast, increased numbers were shown intrahepatically, likely due to increased migration from the periphery to the location of infection [81]. Furthermore, peripheral monocyte numbers were reported to be slightly increased in chronic HBV patients, while also intrahepatic $CD14^+$ $CD16^+$ cells and $CD68^+$ cells increased in frequency correlating with serum ALT levels and/or histological activity index, which are both measures for liver injury. This indicates that, like suggested in chronic HCV and LCMV infection, there might be an increase in infiltrating macrophages and/or a local expansion of KC in chronic HBV [82-84]. Additionally, deficiencies in APC function may contribute to a weak innate and consequently adaptive immune response in chronic HBV patients. Functional deficits

in DC or DC precursors have been reported. Minor phenotypical and functional alterations have been shown in chronic HBV patient-derived mDC compared to those derived from HC. TLR-stimulated mDC of chronic HBV patients produced less TNF and IFNβ than mDC from healthy controls. However the production of IL-6, IL-1β, and IL-12 was similar [85]. Altered cytokine responses possibly affect the T-cell-stimulatory capacity of mDC. Correspondingly, it was shown that mDC from chronic HBV patients are less efficient in inducing T cell proliferation than mDC from healthy controls [85, 86], although Tavakoli *et al.* challenged these findings [87]. Also impaired function of pDC from chronic HBV patients has been described. As mentioned, IFNα is a hallmark and pivotal part of the antiviral immune reaction to virus infection, but HBV was found not to activate IFN production by pDC [55]. Moreover, the capacity of HBV patient-derived pDC to produce IFNα has been attenuated, showing less IFNα production upon stimulation with various stimuli than pDC from healthy controls [80, 81, 85, 88, 89]. Together, this possibly attributes to the absence of IFN-signaling during HBV infection [37, 39]. Correspondingly, HBV and its proteins were shown to be able to cause impaired APC function *in vitro* [55, 62, 90]. Besides being activated by viral proteins and HBV virions, monocyte and KC functions can be attenuated by HBsAg, HBeAg, or hepatitis B virions leading to the inhibition of TLR-induced cytokine production [52-54, 60] as further described in chapter 2 of this thesis. Taken together, there are indications that some deficiencies in APC numbers may underlie an inadequate antiviral immune response against HBV. However, questions like in what way specific impairments contribute to the problem and what the underlying mechanism is remain to be answered in order to identify the factors involved in the impaired immune response towards HBV.

Aim and outline of this thesis

This thesis focuses on specific aspects of the role of APC in chronic HBV infection. Several studies emphasize the importance of APC in the initiation and regulation of the antiviral immune response. Correspondingly, we hypothesize that APC play a pivotal role in the antiviral immune response against HBV. However, what the role of each APC and its subsets is in HBV infection remains to be further specified. IFN are used as antiviral therapy in chronic HBV infection, but what the exact effects of IFN on APC are and how this influences the role of APC in HBV infection remains elusive. Furthermore, APC are constantly exposed to viral proteins, but reports on their interaction with APC are scarce while literature on their effects on APC seem contradictive and/or insufficient. Therefore, our aim is to investigate the effects of IFN and viral proteins on APC in chronic HBV infection to further define the role of APC in HBV infection.

In **chapter 2** we describe the role that KC play and might play in chronic hepatitis B and C infections. Subsequently, part I (comprising chapter 3 and 4) of the original research addresses some effects of *in vivo* and *in vitro* IFN on APC. In **chapter 3** the effects of treatment of chronic HBV patients with IFN monotherapy vs IFN/ribavirin combination therapy are studied on various immune parameters. In **chapter 4**, the immune effects of the novel type III IFN are studied *in vitro* by assessing its effect on NK cells and macrophages. In part II (chapter 5 and 6) we investigate the effect of viral proteins on the immune response. In **chapter 5** the effects on monocytes of *in vivo* exposure to viral proteins and *in vitro* exposure to HBsAg are assessed functionally. In **chapter 6** we investigate KC from chronic HBV patients for presence

of HBsAg and for their phenotype, and further assess the effect of HBsAg on the phenotype and function of KC and monocyte-derived macrophages.

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The role of Kupffer cells in hepatitis B and hepatitis C virus infections

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Chapter 2

Abstract

Globally, over 500 million people are chronically infected with the hepatitis B virus (HBV) or hepatitis C virus (HCV). These chronic infections cause liver inflammation, and may result in fibrosis/cirrhosis or hepatocellular carcinoma. Albeit that HBV and HCV differ in various aspects, clearance, persistence and immunopathology of either infection depends on the interplay between the innate and adaptive responses in the liver. Kupffer cells, the liver-resident macrophages, are abundantly present in the sinusoids of the liver. These cells have been shown to be crucial players to maintain homeostasis, but also contribute to pathology. However, it is important to note that especially during pathology, Kupffer cells are difficult to distinguish from infiltrating monocytes/macrophages and other myeloid cells. In this review we discuss our current understanding of Kupffer cells, and assess their role in the regulation of anti-viral immunity and disease pathogenesis during HBV and HCV infection.

The characteristics of Kupffer cells

Kupffer cells (KC) are tissue-resident macrophages residing in the liver. They are located in the liver sinusoids, and are the largest population of innate immune cells in the liver [1-3]. Due to their abundance and localization, KC are crucial cellular components of the intrahepatic innate immune system that are specialized to perform scavenger and phagocytic functions, thereby removing protein complexes, small particles and apoptotic cells from blood [1-3]. Together with the sinusoidal endothelial cells, KC are the first barrier for pathogens to enter the liver via the portal vein [4]. This is extremely important, since venous portal blood is rich in pathogen-derived products, such as lipopolysaccharide, and pathogens from the gut, which need to be eliminated from the circulation to avoid systemic immune activation.

The specialized function of KC is reflected by the phenotype: they were identified in the early 1970s as peroxidase-positive cells with cytoplasm containing numerous granules and vacuoles, and occasional tubular, vermiform invaginations [5-8]. At present, human KC are identified by immunohistochemistry or flow cytometry using antibodies directed against CD68, CD14 and CD16 [9-11]. However, it is important to mention that these markers are not unique for human KC and macrophages from other tissues, but are also expressed on monocytes, which are also considered a source of precursor cells for KC, and/or dendritic cells [12]. Different from their human counterpart, rat KC are commonly identified by antibodies against CD68 or CD163 (ED1 and ED2, respectively) [13], and mouse KC using the F4/80 marker [14]. However, also the rat and mouse markers are not unique for KC, but are shared with other leukocytes.

The ambiguity in the identification of KC that exists under steady state conditions is even more challenging under pathological conditions in which cellular infiltrates are observed consisting of inflammatory monocytes and/or dendritic cells that share certain surface markers. In rat studies, large and small KC were shown to be present in distinct area within the liver, i.e. in the peri-portal, and peri-venous and mid-zonal area, respectively [10, 15-19], and 2 subpopulations of KC have been isolated from rat liver tissue: $ED1^{+}ED2^{-}$ and $ED1^{+}ED2^{+}$ cells [16, 17]. Similarly, some studies have identified 2 subpopulations of mouse KC: $F4/80^{+}CD68^{+}$ and $F4/80^{+}CD11b^{+}$ cells from mouse liver tissue [20]. It is likely that these populations either illustrate distinct differentiation phases rather than distinct KC subpopulations, or that they identify infiltrating monocytes instead of resident tissue macrophages. In studies from our group, we defined only one KC population in mouse liver tissue on the basis of F4/80 and CD11b expression [21]. This was in line with a study in humans where only a single population of KC was identified as $CD14^{+}$, $HLA\text{-}DR^{+}$, $HLA\text{-}ABC^{+}$, $CD86^{+}$ and $DC\text{-}SIGN^{+}$ cells, with low expression of CD1b, CD40 and CD83 [9]. It is preferable to identify KC not solely based on the available markers, but also on their morphology and phagocytic ability as their hallmark function. In this review, KC are identified as $CD68^{+}$, $CD14^{+}$ and/or $CD11b^{+}$ cells (human), $ED1^{+}$ and/ or $ED2^{+}$ cells (rat) and $CD68^{+}$, $F4/80^{+}$ and/ or $CD11b^{+}$ cells (mouse), according to the original studies. Under steady state condition, the majority of tissue-resident macrophages in the mouse liver have a yolk sac origin and are self-maintained. Upon serious challenge, tissue resident KC can be replaced by precursor cells from bone marrow as well as monocytes, which develop into tissue-resident macrophages [22]. Since the distinction between tissue-resident KC and tissue-infiltrating monocyte/macrophages is difficult, and

since most studies did not discriminate between these cells with a different origin, we will use the term “KC” to describe both cells.

Studies on human KC are being performed using cells obtained from liver tissue or from liver graft perfusate. Liver graft perfusate is preserved in a different manner than liver tissue. Also, tissue-derived KC are commonly isolated using collagenase, a processing step not included for perfusate, which increases the amount of extracellular debris and may induce phenotypic and functional changes. The source of liver material as well as the method to process the samples are important to take into account when interpreting results on the phenotype and function of KC from the various studies.

Macrophages are specialized in sensing and responding to pathogens and equipped with specific pattern recognition receptors, including scavenger receptors, Toll-like receptors (TLR), RIG-like receptors (RLR), NOD-like receptors (NLR) and C-type lectins. These receptors are expressed by tissue-derived as well as *in vitro*-generated macrophages (reviewed in [23]). However, only few of them have been described for KC and it is not clear whether the others are expressed by KC. Scavenger receptors and C-type lectins are important receptors mediating phagocytosis, which are expressed by human, rat and mice KC [24-26]. The phagocytic ability of human KC has been shown in relation to removal of erythrocytes, apoptotic cells and debris [27, 28]. In line with that notion, we and others have shown that rat and mouse KC are strongly phagocytic and possess a high level of basal reactive oxygen species (ROS) production [20, 21]. Upon *in vivo* administration of dextran particles, E. coli or gadolinium chloride, rat and mouse KC take up these particles, produce high levels of ROS, and demonstrate high lysosomal activity [17, 18, 20, 21]. Human KC were shown to express TLR2, TLR3 and TLR4 [9, 29]. The expression of other TLR, as well as NLR and RLR have not been described, but cannot be excluded since the murine counterparts were found to express functional TLR1-TLR9 and RIG-I [25, 30]. In humans and rodents, ligation of TLR on tissue-derived and *in vitro*-generated macrophages resulted in cytokine production [31]. However, to date, studies on the ability of KC to produce cytokines upon TLR ligation resulted in divergent conclusions. For instance, we and others show that KC from human liver tissue and perfusate release IL-10, IL-1β, IL-6, IL-12, IL-18 and TNF upon TLR2, TLR3 and TLR4 ligation ex vivo [9, 32, 33] and [Boltjes, unpublished data]. Similarly, Kono et al showed that liver tissue-derived rat KC produce superoxide, TNF and IL-6 upon TLR4 ligation ex vivo [17]. However, examination of mouse KC isolated from liver tissue by our group and others demonstrated weak induction of TNF and IL-12p40 upon ex vivo stimulation with agonist for TLR4, TLR7/8 or TLR9 [20, 21], whereas no data are available on the cytokine-producing ability of liver perfusate-derived rat or murine KC. Thus, more studies using highly purified KC with a well-defined phenotype need to be conducted to obtain conclusive data on the TLR responsiveness of KC.

A weak ability of KC to produce cytokines might be related to their tolerogenic function in a steady state condition. KC are frequently exposed to gut-derived antigens. Instead of exerting inflammatory responses, human and murine KC constitutively express TGFβ and PD-1, possess high levels of negative regulators downstream the TLR pathway and secrete IL-10 upon LPS stimulation [20, 21, 32, 34-36]. More importantly, the ability of murine KC to produce pro-inflammatory cytokines upon TLR4, TLR7/8 and TLR9 is by far weaker than that of peritoneal macrophages [21]. This observation suggests that KC play a crucial role in maintaining liver homeostasis in a steady state condition. Additionally, our mouse study and others show that

KC are superior in the ability to take up particles and have a higher basal ROS production, in comparison to splenic and peritoneal macrophages, which highlight their function to remove particulates from the circulation [21, 37].

Key Points

- Kupffer cells contribute to immune activation and anti-viral immunity upon infection with HBV or HCV
- Both HBV and HCV are able to exploit the function of Kupffer cells
- The receptors and molecular mechanisms involved in the interaction between Kupffer cells and HBV or HCV, or its components, need to be elucidated
- Kupffer cells and/or liver-infiltrating macrophages contribute to tissue damage and play a role in the regulation of fibrosis, cirrhosis, and hepatocellular carcinoma during chronic viral hepatitis
- The contribution of liver-resident Kupffer cells *vs.* liver-infiltrating macrophages in the regulation of viral immunity and disease pathogenesis is hampered by the lack of distinctive phenotypical markers

The role of KC during LCMV infections

Besides their barrier [4] and janitor function [38, 39], KC have been shown to play a role in the response to pathogens, including viruses. Studies on the importance and anti-viral immune functions of KC in HBV and HCV infections are difficult to perform, since these viruses only infect and replicate in humans and non-human primates, and immunocompetent small animal models for viral hepatitis are not yet available (reviewed in [40, 41]). As an alternative approach several mouse infection models, including lymphocytic choriomeningitis virus (LCMV), murine cytomegalovirus (MCMV), mouse hepatitis virus (MHV) and adenovirus models, have provided information on the role of KC in viral infection. However, in contrast to HBV and HCV where infection and replication is restricted to hepatocytes, these hepatitis mouse models also infect other cells and even other organs. Of these models, MHV and LCMV have been shown to replicate in KC [42, 43]. LCMV, MHV and adenovirus particles can be taken up from the circulation by murine KC via scavenger and complement receptors, which may limit infection [44-47]. It has been shown that failure in clearing LCMV, MHV and adenovirus particles during the acute phase results in “spill-over” infection of hepatocytes, prolonged infection and exacerbated immunopathology [47-49]. Studies using these mouse models have been instrumental in our understanding of the effects on KC during the early phases of virus infections. A number of studies have also evaluated KC during persistent infection in mice. These studies are conducted using specific isolates of LCMV, the clone 13 and WE strains. The development of persistent infection with a high rate of replication of LCMV is similar to HBV and HCV, and important mechanistic pathways identified in LCMV infected mice, were later confirmed to be operational during chronic viral infections in patients. However, in contrast to HBV and HCV, murine LCMV infections are not restricted to the liver, and LCMV replication can also be found in the spleen, lung and kidney. The long-term consequences of human viral hepatitis, such as fibrosis, are absent in mice, although virus-induced liver damage is observed [44, 50]. The effect of chronic LCMV infection on NK cells and virus-specific T cells has been extensively examined, however only few studies have

focussed on KC. In contrast to HBV or HCV, active replication of LCMV in the liver, as evidenced by the detection of viral RNA and antigen, has been demonstrated in KC as well as in hepatocytes [43, 51, 52]. During the first 2 weeks following LCMV infection, an increase of the number of F4/80$^+$ cells is observed, followed by normalization of their numbers [19]. Although differences in MHC class-I expression levels were observed within the F4/80 population by immunohistochemistry, the relative contribution of infiltrating monocytes versus enhanced activation of resident KC is difficult to determine.

An elegant study by Lang *et al.* showed that chlodronate-mediated depletion of KC resulted in rapid LCMV dissemination due to the inability to capture virus, which led to replication within hepatocytes and subsequently severe CD8$^+$ T cell-mediated liver damage [44]. The study further showed that KC responded to type I IFN by inducing the expression of interferon-stimulated genes, and that mice lacking IFNAR specifically on macrophages exhibited strongly enhanced viral titers. However, recently a detrimental influence of granulocytes and macrophages in spleen and liver was reported by their ability to produce reactive oxygen species (ROS) following viral infection, although ROS production by liver F4/80$^+$ cells was low [53]. Importantly, the effect of ROS was an impairment of the immune response, and in the absence of ROS mice exhibited lower viral titers and less liver damage. In a different experimental mouse model, which makes use of transgenic intrahepatic expression of the HBV large envelope protein, ROS activity was observed in KC, and these mice exhibited a chronic necroinflammatory liver disease, resembling human chronic active hepatitis [54].

The findings from the LCMV mouse model clearly show the complexity of the anti-viral response in the liver since KC can both contribute to promote and suppress viral eradication and liver pathology. In the following section, we will focus on the interaction of KC with HBV and HCV, and the functional consequences.

The role of KC during HBV and HCV infections

Both HBV and HCV are transmitted predominantly via percutaneous and sexual exposure, while perinatal exposure is often seen for HBV only [55-57]. Infection with these viruses can either resolve spontaneously or develop into chronic liver disease with continuous viral replication in hepatocytes [56-58]. Chronic hepatitis poses an increased risk for liver fibrosis and cirrhosis, hepatic failure, and hepatocellular carcinoma (HCC) [58, 59]. Patients with a self-limiting HBV or HCV infection show sustained, vigorous and multi-epitope-specific CD4$^+$ or CD8$^+$ T cell and B cell responses, whereas in chronic HBV and HCV these responses are weak and/or transient [60-63]. This demonstrates that clearance of the infection is dependent on strong multi-epitope-specific T and B cell responses, which is only possible following effective innate immune responses [63, 64]. Here, we will firstly address the role of KC in the interaction and recognition of HBV and HCV, and their role in the induction of a pro-inflammatory response. Pro-inflammatory mediators are important for inhibition of viral replication, the induction of resistance to infection of neighboring cells, and attraction and activation of other immune cells, and consequently contribute to the development of effective virus-specific immunity. Secondly, we will discuss KC-virus interactions that may inhibit the development of effective viral immunity, facilitate

viral persistence or promote liver damage.

Interaction of KC with HBV and HCV

HBV is a 3.2 kb partially double-stranded DNA envelope-virus which replicates via RNA intermediates. Hepatitis B core protein (HBcAg)-encapsulated viral DNA and hepatitis B envelope protein (HBsAg) form a complete viral or Dane particle. HBV particles, HBsAg, and hepatitis B early antigen (HBeAg; a truncated form of HBcAg) are secreted by infected hepatocytes and can be detected in serum of HBV patients [58, 65].

Evidence for productive HBV infection of cells other than hepatocytes is lacking. Also, detailed information on the presence of HBV (proteins) in KC *in vivo* or the uptake of HBV or its proteins by human KC ex vivo has not been reported. Although no information is available on KC, studies using THP-1 monocytic cells, monocytes and dendritic cells have shown binding of HBV or HBV proteins, leading to their activation. For instance, TLR2 and heparan sulfate proteoglycan (HSPG) were suggested to be responsible for HBcAg recognition on THP-1 cells, and HBcAg-induced activation of THP-1 cells resulted in production of IL-6, IL-12p40, and TNF [66]. However, since HBcAg is only found within infected hepatocytes or viral particles, it is unclear whether HBcAg interacts with KC, via HSPG and/or another extracellular receptor like TLR2. Also, other receptors expressed by KC are known to interact with HBV proteins as demonstrated in other cell-systems (Table 1). For instance, HBsAg can interact with human blood monocytes in a CD14-dependent fashion [67], and with dendritic cells via the mannose receptor [68], which are both receptors known to be also expressed on KC [69]. Finally, complex formation of HBsAg with albumin may lead to enhanced uptake of HBsAg from the circulation by KC and endothelial cells [70].

HCV contains a 9.6 kb positive-strand RNA genome that translates into the structural proteins, core and E1 and E2 envelope proteins, and the non-structural proteins NS1-NS5. After replication, they form a small-enveloped virus particle containing the newly synthesized RNA genome [71, 72].

Compared to HBV, there is a better understanding of the entry receptors on hepatocytes used by HCV. In addition to claudin1, occludin, epidermal growth factor receptor (EGFR) and ephrin type-A receptor-2, HCV infects hepatocytes by attaching to HSPG, low-density lipoprotein (LDL) receptor, scavenger receptor (SR)-B1 and CD81. Some, but not all, receptors are expressed by KC (Table 1) [73-82]. It has been reported that incubation of human liver cells with HCV-E2 resulted in HCV-E2 binding to KC in a CD81-dependent manner [83], but also DC-SIGN, a C-type lectin not expressed by hepatocytes, has been demonstrated to bind HCV on KC [84-86].

Although it is unlikely that HCV can replicate in KC, activation of KC by HCV and its proteins has been demonstrated. HCV core and NS3 stimulate human liver perfusate-derived $CD14^+$ KC and monocyte-derived macrophages via TLR2 to produce pro-inflammatory IL-1β, IL-6, and TNF and immunosuppressive IL-10 [84, 87]. Recently, it was shown that TLR4, in density gradient- and adherence-isolated liver-derived human KC, mediates NS3 recognition, resulting in TNF production [88]. However, HCV core and NS3 are not secreted at significant levels by infected hepatocytes, posing little relevance to extracellular recognition of HCV by KC via

Table 1: Surface molecules and secreted inflammatory mediators facilitating KC roles in HBV/HCV infection.

	HBV		HCV	
	Mediators	**Reference**	**Mediators**	**Reference**
Binding/Uptake				
	HSPG	79	HSPG	79
	CD14	9	SR-B1	82
	mannose receptor	69	LDL-receptor	81
			DC-SIGN	9, 85
Pattern Recognition Receptors				
			TLR2	84, 87
			TLR4	88
Cytokines				
	IL-1β	89	IL-1β	84, 100
	IL-6	89	TNF	84
	TNF	89	IL-10	84
	TGFβ	91		
Chemokines				
	CXCL8	89		
Co-stimulatory molecules				
			CD40	94
			CD80	94
			MHC class II	94
Immune inhibition or promotion of tolerance				
	TGFβ	91	PD-L1	84, 137
	PD-L2	30	IL-10	84
	galectin-9	135	galectin-9	120
Liver damage				
	IL-6	89	TRAIL	84
	TRAIL	84	granzyme B	105, 106
	FasL	106	perforin	105, 106
	granzyme B	105		
	perforin	105		
	ROS	54		
	galectin-9	135		
	TGFβ	91		

these TLR. Alternatively, phagocytosis of infected hepatocytes by KC may allow intracellular exposure to viral RNA, but so far no evidence exists.

Stimulatory effects of HBV or HCV on KC function

There are only few publications that show a stimulatory effect of HBV or HBV proteins on the function of KC. Hösel *et al.* showed that HBV particles and HBsAg induce IL-1β, IL-6, CXCL8 and TNF production by human $CD68^+$ cell-enriched non-parenchymal cells via NF-□B activation [89] and subsequently inhibit HBV replication in primary hepatocytes. This inhibitory effect was mainly ascribed to IL-6, but also TNF inhibited HBV replication in a non-cytopathic manner [90]. In contrast, Li *et al.* demonstrated that rat $ED1^+$ adherent KC exposed to HBV virions hardly expressed

IL-1β, IL-6 or TNF, but produced the immunoregulatory cytokine TGFβ [91].

During chronic HCV infection, KC are increased in numbers in the liver [92, 93], and exhibit an activated phenotype with higher mRNA expression levels of the activation markers CD163 and CD33 in livers of chronic HCV patients versus controls [94, 95]. Recently, it was reported that in response to HCV human KC release IL-1β and IL-18 *in vitro* [96]. In line with these findings, stimulation of $CD14^+CD68^+$ cells from liver perfusate with UV irradiated cell culture-derived HCV induced IL-1β production. To support this data, *in vivo* co-expression of IL-1β and CD68 was observed using immunofluorescence on liver tissues from patients with chronic HCV [97]. Besides intrahepatic IL-1β, also elevated serum IL-1β levels were detected in patients as compared to healthy individuals [97].

Although a direct effect of HCV-exposed KC on HCV replication is unknown, it was recently reported that KC-derived TNF increased the permissivity of hepatoma cells to HCV. In this study, LPS as well as HCV induced KC to produce TNF, thereby indirectly promoting HCV infection [33]. On the other hand, HCV- or TLR-ligand-induced KC-derived cytokines, such as IL-6, IL-1β, and IFNβ [84, 87, 97, 98], were found to inhibit HCV replication in the HCV replicon model [98-100], implying that KC are also capable of displaying antiviral activity upon HCV exposure.

In addition, release of chemokines and cytokines by KC has an indirect effect on the immune response in the liver by recruitment and activation of infiltrating leukocytes, as also discussed by Heydtmann *et al.* [101]. This may result in a complex interaction between factors produced by liver parenchymal cells, liver resident immune cells including KC, and infiltrating leukocytes. KC are able to activate NK cells and NKT cells, both present at relatively high numbers in the liver, via the production of pro-inflammatory cytokines [9]. In turn, NK and NKT cells produce cytokines such as TNF and IFNγ and are cytotoxic in nature [9, 102]. Upon HBV exposure, KC were found to produce CXCL8 [89], which potentially attracts NK and NKT cells during the early phase of HBV infection. KC are also able to recruit dendritic cells to the liver, which involved C-type lectins interactions [103]. This enhanced dendritic cell recruitment may initiate and promote virus-specific T cell responses. In contrast to dendritic cells, KC are less efficient in priming naïve T cells. Nevertheless, mouse KC have been shown to present antigen to $CD4^+$ and $CD8^+$ T cells, inducing these to proliferate and produce IFNγ [104, 105]. The relatively high expression of CD40, CD80 and MHC class II found on $CD68^+$ cells in chronic HCV patients [94] might point towards possible antigen presentation by intrahepatic macrophages.

Although lymphocytes such as NK cells and $CD8^+$ T cells are potent effector cells responsible to kill virus infected cells, KC have been reported to express cytotoxic molecules such as TRAIL, Fas-ligand, granzyme B, perforin and ROS, enabling them to lyse infected hepatocytes [106-108]. However, since KC act in an antigen-nonspecific manner and hence can lyse hepatocytes irrespective of their infection state, it is tempting to speculate that KC cause more damage to the organ due to their cytotoxic capacity than that they provide protective immunity to the host.

In summary, only limited information exists on the direct interaction between HBV and HCV with KC *in vivo* and ex vivo. Macrophages are able to bind HBV or HCV or virus-related proteins *in vitro*, triggering surface and/or intracellular receptors. However, receptors used for these purposes need to be further investigated. Several studies indicate that KC may play a role in controlling HBV and HCV infections by

inhibiting viral replication, either directly via the production of cytokines or via their interaction with other cells, as well as in shaping the inflammatory response towards the induction of virus-specific immunity. However, more research is required to get a better insight into the role of KC in regulating intrahepatic immunity.

Suppressive effects of HBV and HCV on KC function

Besides the contribution of KC to viral clearance, viruses may actively interfere with the pro-inflammatory functions of KC to evade host immunity. Various studies show that HBV and HCV are able to interfere with TLR pathways, RIG-I signaling and subsequent pro-inflammatory activities of hepatocytes and immune cells [109-113], but studies describing the effect on human KC are limited. Only one study described that type I IFN production and TRAIL expression by human perfusate-derived KC were suppressed by HCV core protein via disruption of the TLR3/TRIF/TRK1/IRF3 pathway [84]. In addition, numerous studies on monocytes have demonstrated modulation of cytokine production by HCV proteins, and altered TLR responsiveness of monocytes obtained from chronic HCV patients [114-116].

Concerning HBV, pretreatment of non-parenchymal cells including KC, with HBV-Met cell-derived supernatants, HBsAg, HBeAg or hepatitis B virions almost completely abrogated TLR-induced anti-viral activity, i.e. IFNβ production, interferon-stimulated gene (ISG) induction, IRF3, NF-□B, and ERK1/2 expression [117]. Accordingly, incubating human monocytes with HBeAg or HBsAg inhibited TLR2-induced phosphorylation of p38 MAPK and JNK MAPK, and subsequent production of IL-6, TNF, and IL-12 [29, 118, 119]. *In vivo*, TLR2 expression by KC and peripheral blood monocytes in HBeAg-positive chronic HBV-infected individuals was lower than that in HBeAg-negative patients and controls. Moreover, TLR2 ligation induced less IL-6 and TNF in those HBeAg-positive patients [29]. These alterations may be related to the inhibitory effect of HBeAg on TLR2 signaling demonstrated *in vitro*. In addition, also TLR3 expression was found to be lower on PBMC from chronic HBV patients compared to control patients as well as on liver cells, including KC [120]. Antiviral therapy of chronic HBV patients with entecavir or pegylated IFNα partially restored TLR3 expression, but it is unclear whether this is a direct viral effect.

Tolerogenic effects of HBV and HCV related to KC

As mentioned above, KC are constantly exposed to pathogen-derived products from the gut. To prevent excessive inflammation and pathology of the liver, continuous activation of KC is avoided as these cells become refractory to subsequent endotoxin challenge, a phenomenon known as endotoxin-tolerance [121, 122]. This contributes to the well-described tolerogenic milieu in the liver. Besides modulation of TLR-signaling pathways, also expression of anti-inflammatory mediators, such as IL-10 and TGFβ, and other soluble and membrane-bound inhibitory molecules are underlying the intrahepatic tolerance [35, 105, 122, 123].

A number of studies have reported that HBV and HCV components affect the production of immunoregulatory cytokines, and consequently promote the tolerogenic milieu of the liver. In this respect, it has been reported that HBV particles preferably induced TGFβ production by rat KC instead of pro-inflammatory cytokines [91]. One

of the activities of TGFβ is that it plays a role in maintaining tolerance towards self-antigens by selectively supporting the differentiation of FoxP3$^+$ regulatory T cells [124, 125]. Furthermore, HCV core protein induces IL-10 production by human KC [84, 87]. Elevated intrahepatic IL-10 levels may suppress pro-inflammatory cytokine production by intrahepatic cells, frustrate KC-NK cell interaction [9, 126] and antigen presentation to T cells and their activation [105, 127-133]. Interestingly, chronic HBV and HCV patients showed higher plasma levels of IL-10 than uninfected individuals [134, 135], which could be the result of a direct viral effect on KC and/or other cells, or the result of a negative feedback mechanism resulting from ongoing liver inflammation. Recently, the role of KC was examined in an established HBV-carrier mouse model. In this model, KC as well as IL-10 were involved in the establishment of antigen-specific tolerance towards peripheral HBsAg vaccination [136].

KC express membrane-bound inhibitory ligands that could facilitate a tolerogenic milieu in the liver. For instance, under steady state conditions, KC are known to express PD-L1, which is a ligand for PD-1 and known to impede T cell function by inhibiting proliferation and cell division [36]. Immunohistochemical analyses of liver biopsies from chronic viral hepatitis patients revealed that CD68$^+$ macrophages expressed increased levels of PD-L2 compared to control liver tissue [30, 123, 137]. Similar results were reported for galectin-9 with enhanced expression by CD68$^+$ cells by immunohistochemistry, which was confirmed by flow cytometry [137]. Interestingly, enhanced serum levels of galectin-9 were observed in patients with biochemical evidence of highly active chronic HBV-related liver disease (ALT>100 U/L) as compared to patients with relatively low ALT levels (<50 IU/L) or healthy controls. Also comparison of plasma galectin-9 levels in patients with chronic HCV showed higher levels in patients compared to healthy individuals [123], Furthermore, co-localization of CD68 and galectin-9 was observed in the peri-portal regions of the livers of virtually all the patients with HCV infection, regardless of grade of inflammation or stage of fibrosis, but not in normal control livers [123].

These inhibitory ligands are known to inhibit T cell function upon cell-cell contact via interaction with PD-1 and Tim-3, respectively [138], which is of relevance since both PD-1 and Tim-3 are reported to be upregulated on HBV- and HCV-specific intrahepatic and peripheral blood-derived CD8$^+$ T cells and associated with T cell dysfunction and exhaustion during chronic viral hepatitis [123, 139, 140]. Intrahepatic expression levels of PD-L1, PD-L2 and PD-1 correlated with liver inflammation in chronic HBV [30]. Although it has been shown that HCV core protein can induce PD-L1 expression on human perfusate-derived KC [84], it is not clear whether the upregulation of inhibitory ligands on intrahepatic macrophages and its correlation with inflammation are direct effects of HBV or HCV, or are components of negative feedback mechanisms that develop as a consequence of persistent inflammation.

Thus, several studies indicate that both HBV and HCV compromise anti-viral immunity to a certain extent by (1) interfering with signaling of pathogen recognition receptors and the production of pro-inflammatory cytokines by KC and (2) increasing the tolerogenic capacities of KC resulting in the elevated expression of anti-inflammatory mediators. As persistent inflammation in general is accompanied by negative feedback mechanisms, the KC-related anti-inflammatory signals observed during chronic viral hepatitis could be explained by direct viral effects, immune regulation as part of the ongoing inflammatory response, or a combination. However, also immune

activating functions of KC have been described upon HBV/HCV interaction. These seemingly contradictory functions probably indicate a critical balance influenced by the extent to which receptors are triggered (or over-triggered) and also by the type of KC receptors that are triggered. Therefore, not only the concentration of virus (proteins), but also the time since infection may strongly affect KC function. Whether also age influences KC function as one of the mechanisms explaining the self-limiting hepatitis often seen in HBV-infected adults, whereas young children usually develop chronic infection, has to be investigated.

Role of KC in viral hepatitis-related liver damage

Liver fibrosis

One of the consequences of sustained low-grade injury induced by persistence of HBV and HCV in the liver is fibrosis, which is characterized by excess collagen deposition and accumulation of extracellular matrix. HBV and HCV may induce fibrinogenesis by activating hepatic stellate cells directly or indirectly by inducing cellular injury, apoptosis and necrosis, which triggers a wound healing response. KC are thought to be involved in fibrogenesis by the release of various pro-fibrinogenic factors, such as ROS and certain cytokines, such as IL-6, TNF, IL-1, PDGF and TGFβ, that induce activation of hepatic stellate cells [141]. In addition, KC produce enzymes that are important or the breakdown of matrix, such as collagenases and metalloproteinases, but they also regulate the production of these factors by other cells, leading to disturbance of the homeostatic mechanisms involved in extracellular matrix deposition [142]. Recent studies in experimental animal models demonstrate that these activities are only partially conducted by liver-resident macrophages, but largely depend on recruitment of monocytes as precursors of macrophages into the inflamed and damaged liver [143, 144].

Although, in patients with viral hepatitis, no causative role has been demonstrated for KC in the development of liver fibrosis, increased numbers of $CD14^{+}CD68^{+}$ KC were found around the regions of damage and fibrosis [134]. These increased numbers were associated with liver injury [93, 141, 145, 146]. A detailed study by Liaskou *et al.* observed that in liver tissue from non-viral hepatitis patients with end-stage liver disease a specific monocyte subpopulation accumulated in the liver, which was able to conduct phagocytic activity and to release inflammatory and profibrinogenic cytokines [147]. Interestingly, a study in HBV replication-competent transgenic mice showed an opposite effect of KC by demonstrating that they did not contribute to liver damage, but prevented liver injury by removal of apoptotic hepatocytes during viral hepatitis [39]. In this model, clodronate-mediated depletion of KC resulted in higher numbers of necrotic hepatocytes and elevated serum ALT levels. In line with this, in a different mouse model, liver-infiltrating monocyte/macrophages mediated regression of fibrosis via phagocytosis of cellular debris [148].

Liver damage and ultimately the induction of fibrosis may be, at least in part, attributed to cytokines produced by KC. Moreover, during viral hepatitis KC have also been found to express cytotoxic molecules, like TRAIL, Fas-ligand, granzyme B, perforin and ROS, that enable them to kill infected as well as non-infected “bystander” hepatocytes [106-108]. Fas-ligand expression by KC was increased in

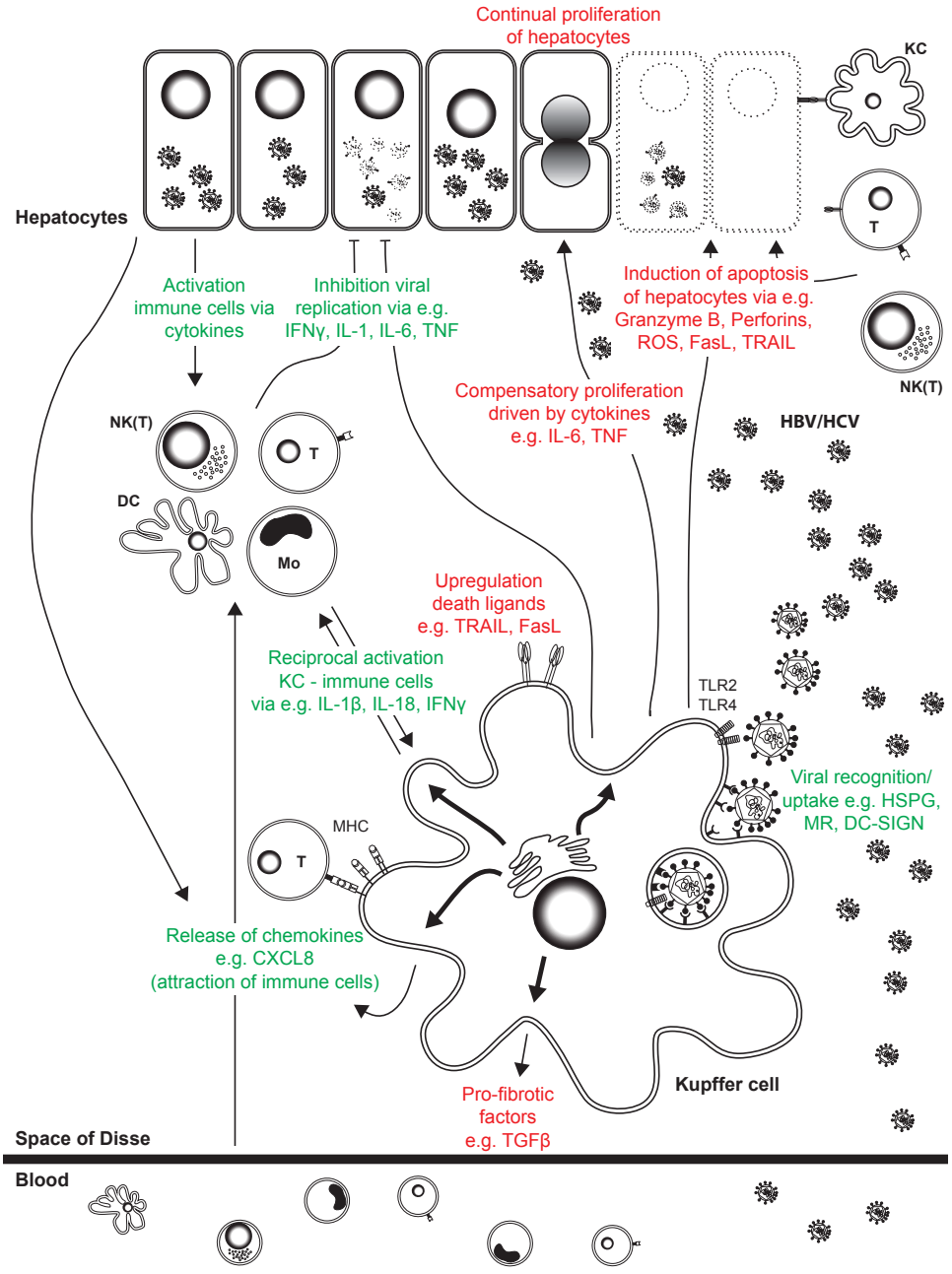

Figure 1. The role of KC in anti-viral immunity and tissue damage during HBV and HCV infection. Exposure of KC to HBV or HCV will lead to direct activation of KC that, together with infected hepatocytes, release cytokines and chemokines, which are responsible for the attraction of other leukocytes. Activation of infiltrating immune cells leads to further production of cytokines that indirectly activate KC. The secreted cytokines may inhibit viral replication (green text). However, persistent exposure of KC to HBV or HCV will continuously activate KC leading to the ongoing release of cytokines and chemokines attracting and activating more leukocytes. Likewise, continuous activation of infiltrating leukocytes leads to ongoing production of cytokines that indirectly activate KC. Some of the cytokines secreted are pro-fibrotic factors.

chronic HBV patients and associated with elevated ALT levels, while granzyme B and perforin expression by KC was increased in both chronic HBV and HCV patients [106, 107]. Interestingly, a direct contribution of KC to the pathogenesis of hepatitis has also been reported for viral infections by viruses that infect other organs and are not detected in the liver itself [149]. In influenza infection, KC were indicated as the effector cells killing hepatocytes in an as yet unidentified manner, leading to damage-associated hepatitis. KC can kill hepatocytes either directly via Fas-dependent apoptotic pathways or indirectly by interacting with $CD8^+$ (and possibly $CD4^+$) T cells through stimulation of cytokine secretion and other mediators, such as ROS [149].

Hepatocellular carcinoma

Chronic HBV/HCV and cirrhosis are major risk factors for the development of hepatocellular carcinoma [150]. Although HCC development has been extensively studied in mice and rat, only few studies have directly assessed the importance of KC in HCC development in chronic HBV setting, and no studies are available from chronic HCV setting. Dying hepatocytes, likely resulting from anti-viral activities since HBV and HCV are considered non-cytopathic, will activate neighboring cells, including KC [151], to produce cytokines and growth factors, such as hepatocyte growth factor, IL-6 and TNF, which will further amplify the inflammatory response and drive the compensatory proliferation of surviving hepatocytes [152]. Ongoing cycles of hepatocyte death and regeneration increase the chances of spontaneous mutations and DNA damage [153] eventually resulting in HCC. In HBV-transgenic mice, KC and/or infiltrating macrophages produced high levels of ROS, resulting in extensive oxidative DNA damage in neighboring proliferating hepatocytes and development of HCC [54]. HBV/HCV also activate KC to produce these types of pro-inflammatory mediators, which may support the development of HCC [84, 89]. Additionally, the immunoregulatory mediators expressed by KC, either as a direct virus-KC interaction or as a consequence of the inflammatory response, may also inhibit tumor-specific immune responses. For instance, galectin-9 expressed on intrahepatic macrophages caused senescence of $CD4^+$ and $CD8^+$ $Tim3^+$ T cells, and may explain part of the mechanism leading to the development of HCC [154]. Furthermore, one of the HBV-derived proteins, HBxAg, also has direct tumorigenic effects [155]. Hepatocyte regeneration, either influenced by KC or not, allows HBxAg integration in DNA of hepatocytes, which is one of the processes involved in the development of HCC (reviewed in [153]). Whether HBxAg directly interacts with KC is not described.

In conclusion, KC play a central role in liver damage during hepatitis, having all the tools to induce inflammation, cell death, fibrosis and ultimately HCC, but further research during HBV/HCV infection remains to be carried out to determine the exact contribution of KC to liver damage in viral hepatitis.

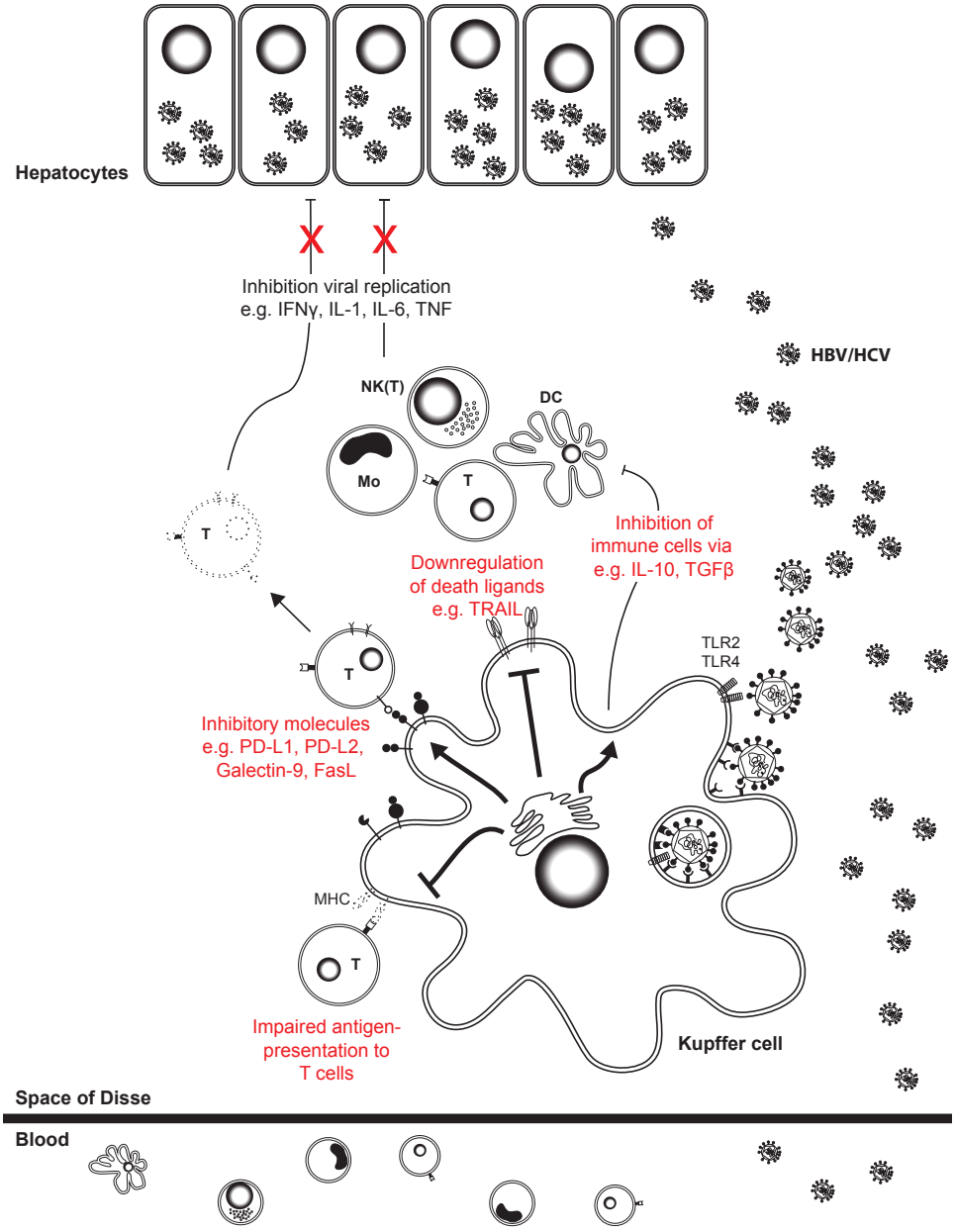

Figure 2. Role of KC in immune regulation and viral persistence during HBV and HCV infection. Exposure of KC to HBV or HCV will lead to their activation and the release of anti-inflammatory cytokines and expression of inhibitory molecules. Combined with impaired antigen presentation by KC, these regulatory mechanisms will interfere with KC function and that of other immune cells, frustrating anti-viral immunity.

Perspectives

Currently, our understanding of the role of KC in viral hepatitis is incomplete. The detailed contributions of liver-resident KC versus liver-infiltrating macrophages to various processes of disease pathogenesis are difficult to determine, because of the highly overlapping characteristics of these cells. Nevertheless, we can appreciate several possible anti-viral roles of KC, including binding and/or uptake of virus leading to immune recognition and the production of pro-inflammatory mediators resulting in (1) inhibition of viral replication in hepatocytes, (2) activation of neighboring cells, and (3) attraction, activation and interaction with other immune cells, which will further increase the anti-viral and inflammatory response (Fig. 1.). These immune activating roles of KC are beneficial to combat HBV and HCV in the early phases after infection, but may also contribute to tissue damage and the development of fibrosis, cirrhosis and HCC during chronic viral hepatitis (Fig. 1.). Furthermore, also immune regulatory functions of KC have been described, either as a consequence of direct virus-KC interaction, or as part of the complex tolerogenic liver environment and the ongoing inflammatory response upon HBV and HCV-infection, which may counteract the development of effective anti-viral immunity and support viral persistence and related disease pathogenesis (Fig. 2.).

With our growing appreciation of the roles of intrahepatic macrophages in both protective and harmful responses, intrahepatic macrophages form an interesting but complex cellular target for treatment options in viral hepatitis. The versatile features assigned to KC may partly belong to infiltrating monocytes/macrophages and therefore future efforts should focus on identifying phenotypical and/or functional characteristics discriminating KC from infiltrating macrophages. Furthermore, the function of KC and other intrahepatic macrophages will largely depend on the type, the level and duration of receptors triggered pushing the balance towards either protective or harmful responses. Identification of receptors and underlying molecular mechanisms involved in virus-cell interactions and insight into mechanisms involved in wanted and unwanted responses of the different macrophage populations that exert distinctive functions during the early and later phases of HBV/HCV infection are needed to move the field forward.

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Assessment of the effect of ribavirin on myeloid and plasmacytoid dendritic cells during interferon-based therapy of chronic hepatitis B patients

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Chapter 3

Abstract

The combination of ribavirin and peginterferon is the current standard of anti-viral treatment for chronic HCV patients. However, little is known on the mode of action of ribavirin in the anti-viral treatment of HCV patients. To investigate the immunomodulatory mechanism of ribavirin, we studied peginterferon alone versus peginterferon and ribavirin in chronic HBV patients. The addition of ribavirin did not affect the number of myeloid dendritic cells (mDC) or plasmacytoid dendritic cells (pDC), nor did it enhance T-helper-1 cell activity or T-cell proliferation. In contrast, it increased upregulation of activation markers on mDC and pDC, which was sustained throughout treatment. However, the addition of ribavirin had no effect on IFNα production by pDC. Our findings demonstrate that, although ribavirin does not lead to a viral load decline, *in vivo* treatment with ribavirin affects the activation of pDC and mDC in chronic HBV patients.

Introduction

Chronic infections with HBV and HCV cause serious global public health problems that affect over 500 million people worldwide. Chronicity of these infections is the result of complex and as yet ill-defined interactions between the replicating non-cytopathic virus and inadequate antiviral immune responses [1-3]. Due to complications of chronic viral hepatitis, such as liver failure and hepatocellular carcinoma, yearly over 800,000 deaths occur globally [4].

IFNα is an important antiviral cytokine, which, in its pegylated form, is used as first-line treatment for chronic HBV. Peginterferon leads to sustained viral responses in only 30% of the patients and in only 7-18% of HBeAg-negative patients [5, 6]. For chronic HCV patients, the combination of peginterferon and the nucleoside analogue ribavirin is the current standard of treatment [7]. The addition of ribavirin to the monotherapy with peginterferon to treat patients with chronic HCV infections doubles the sustained viral response rate [7, 8]. The mechanism of action of ribavirin in combination therapy against HCV is still a matter of debate. As well as inhibiting viral replication by interfering with viral mRNA, ribavirin has been reported to modulate the immune response [9, 10]. It has been shown by some groups that ribavirin can promote the development of T-helper 1 (T_H1) cells as shown by enhanced IFNγ production [11-13], while others failed to do so [14]. The effect of ribavirin on dendritic cells (DC) is not entirely clear, because of the use of different cell types from different species. Using human immature monocyte-derived DC stimulated with poly-I:C, addition of ribavirin caused a reduction of the production of cytokine production [15], which could not be explained by enhanced cell death or apoptosis. However, using mouse bone marrow derived DC, the effect of ribavirin was an enhancement of IL-12p40 production accompanied by a reduction of IL-10 production [16]. In this study, cytokine production was induced by ribavirin alone without the additional triggering via TLR or other receptors. No data are available on the *in vivo* effects of ribavirin on human DC.

Previously, we reported that -despite the promising results of earlier studies [17, 18] - the addition of ribavirin to peginterferon treatment in chronic HBV patients did not improve the efficacy of therapy [6]. Since viral load decline, and virological and biochemical responses were comparable in both treatment arms, we now had the opportunity to compare the immunological features between the two groups without any interfering clinical differences.

In our cohort of chronic HBV patients [6] we aimed to better understand the modulation of ribavirin on immunological processes, which can not be studied in chronic HCV patients where ribavirin addition leads to viral decline. We here examined whether the addition of ribavirin to the treatment protocol affected the phenotype or function of myeloid and plasmacytoid DC of HBV patients, as well as their T cell responses. This study contributes to the ongoing discussion on whether ribavirin can enhance innate and adaptive immunity.

Material and methods

Patients

Table 1 shows the patient characteristics of 14 chronic HBV patients who were HBeAg-negative, anti-HBe positive and had been HBsAg-positive for over 6 months. These patients were part of the PARC multicenter study, and received either combination therapy with 180 µg peginterferon α-2a (Pegasys, Hoffmann-La Roche Ltd., Basel, Switzerland) weekly and ribavirin (Copegus, Hoffmann-La Roche Ltd.) 1,000 mg daily (body weight <75 kg) or 1,200 mg daily (body weight ≥75 kg); or monotherapy with 180 µg peginterferon α-2a once per week and placebo. Therapy was for 48 weeks, after which a follow-up period of 24 weeks ensued. As part of the diagnostic evaluation, serum HBV load and alanine aminotransferase levels (ALT) were determined at indicated time points. Heparinized peripheral blood samples were obtained at baseline and after 12, 24, 36, 48 weeks of therapy. A follow up sample was obtained 24 weeks after treatment. The study was approved by the local ethics committee, and all patients in the study gave informed consent before inclusion in the study.

Leukocyte numbers and phenotypes

Myeloid and plasmacytoid DC numbers were determined in fresh whole blood samples by white blood-cell counting and flow cytometry after staining with antibodies against CD123 (BD Pharmingen, San Diego, CA, USA), CD45 (Beckman Coulter, Brea, CA, USA), CD20 (ExBio Nuclilab, The Netherlands), BDCA-1 and BDCA-4 (Miltenyi Biotec, Bergisch Gladbach, Germany). To analyze the frequency of leukocyte subpopulations and expression of chemokine receptors, activation markers, and other phenotypical markers we stained fresh and/or thawed peripheral blood mononuclear cells (PBMC) with antibodies against CD3, CD4, CD8, CD14, CD56, HLA-DR (all BD), CD14 (BD Pharmingen), CD11a, CD16, CD80 (all Beckman Coulter), CCR3, CD86 (both BioLegend, San Diego, CA, USA), CD11c, CD20, CD40, HLA-DR (all eBioscience, San Diego, CA, USA), BDCA-1, BDCA-3, BDCA-4 (Miltenyi), CCR4, CCR5 and CCR7 (all R&D). PBMC were isolated from blood by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) gradient centrifugation. Data was acquired on a FACScalibur or FACScanto (both BD) and analysed with FlowJo (Tree Star, Inc., Ashland, OR, USA) and FACSdiva software (BD).

Intracellular cytokine staining

Thawed PBMC ($1x10^6$ mL-1) were cultured in 48-well plates (Corning, Lowell, MA, USA) in 250 µl RPMI culture medium (Lonza, Verviers Sprl, Belgium) containing 10% heat-inactivated human serum (Lonza), penicillin/streptavidin (Gibco) and L-glutamin (Lonza) for 4 hours at 37°C, 5% CO_2. The cells were either unstimulated or stimulated with 10 ng mL-1 PMA and 0.4 µg mL-1 ionomycin (both Sigma-Aldrich, St. Louis, MO, USA). During the last 3 hours of culture, brefeldin (10 µg mL-1; Sigma-Aldrich) was added. The cells were harvested, fixed with 2% formaldehyde, permeabilized with 0.5% saponin (VWR, West Chester, PA, USA) and stained for IFNγ, IL-4, CD3

Table 1

Patient characteristics

Patient #	Age (yr)	Sex	HBV Genotype	Viral load (geq/ml)	ALT (U/l)	Therapy	Response to therapy
1	34	M	D	$9.0x10^4$	76	Peg	Biochemical
2	31	M	D	$9.5x10^6$	443	Peg	Combined
3	52	M	A	$4.5x10^5$	63	Peg	Poor
4	29	F	E	$3.8x10^7$	109	Peg	Poor
5	35	F	D	$4.3x10^4$	47	Peg	Combined
6	36	M	D	$3.6x10^7$	99	Peg	Poor
7	47	M	D	$7.3x10^3$	183	Peg	Poor [a]
8	35	M	D	$1.8x10^6$	61	Peg	Biochemical
9	32	M	D	$1.3x10^7$	196	Peg/riba	Poor
10	65	M	A	$4.2x10^7$	201	Peg/riba	Virological
11	41	M	D	$7.2x10^6$	110	Peg/riba	Poor
12	63	M	A	$7.1x10^4$	63	Peg/riba	Virological
13	52	F	C	$3.7x10^4$	71	Peg/riba	Combined
14	41	M	C	$2.8x10^6$	61	Peg/riba	Combined

Response to therapy at week 72 (end of follow-up period): Poor response: Viral load $>10^3$ HBV copies/ml and ALT >40 for male, >30 for female; Biochemical response: ALT normalization (<30/40 for F/M, resp.); Virological response: Viral load $<10^3$ HBV copies/ml; Combined response: Viral load $<10^3$ HBV copies/ml and normalization of ALT; [a] Switched to adefovir after 12 weeks; geq = genome equivalents

(all BD) and CD4 (eBioscience). Where appropriate, isotype antibodies were used as controls. Data was acquired and analyzed as above.

Proliferation assay

To analyze the proliferation of T cells, thawed PBMC were cultured in quadruplicate in round-bottom 96-well plates (Corning) in a final concentration of $1x10^6$ cells per ml in 200 µl RPMI culture medium at 37°C, 5% CO_2. On day 0, cells were either unstimulated or stimulated with HBcAg (1 µg mL-1; American Research Products, Belmont, MA, USA), CMV (35 µg mL-1; Microbix Biosystems, Toronto, Ontario, Canada) or antibodies against CD3 (400 ng mL-1; Orthoclone OKT®3; Janssen-Cilag, Tilburg, the Netherlands) and CD28 (1 µg mL-1; BioLegend). At day 5, the cells were pulsed for 16 hours with 0.5 µCi per well [3H]-Thymidine (Amersham, Little

Chalfont, UK). Proliferation was determined by liquid scintillation and expressed as counts per minute (cpm).

Statistics

To compare therapy groups, peginterferon treatment was analyzed against peginterferon and ribavirin treatment at the same time point. These data were analyzed using the non-parametric Mann–Whitney U test. Data from time points during therapy and during follow-up were pair analyzed against start of therapy, and end of follow up was pair analyzed against end of treatment. These data were analyzed using the non-parametric Wilcoxon Signed Ranks Test with two-tailed P-values. Data from cytokine expression are normalized and given as percentages of the appropriate control. Data on expression of costimulatory molecules, chemokine receptors and adhesion molecules are expressed as geometric mean ± SEM. A P¬-value of < 0.05 was considered statistically significant.

Results

Frequency of DC populations decreases during peginterferon-based therapy in chronic HBV patients

To investigate the effect of peginterferon monotherapy and combination therapy with ribavirin on circulating antigen-presenting cells, peripheral blood samples were assessed for the frequency and numbers of DC populations. As a consequence of therapy, there was a reduction in the absolute leukocyte numbers (data not shown). The absolute cell numbers and frequency of BDCA-1$^+$ myeloid DC (Figure 1) and BDCA-3$^+$ myeloid DC (Supplementary Figure S1) decreased during therapy, irrespective of whether peginterferon alone or peginterferon in combination with ribavirin was given. However, the absolute and relative numbers of BDCA-4$^+$ plasmacytoid DC did not change during either therapy. Additionally, albeit not significant, the frequency of total monocytes showed a decline during therapy, which was accompanied by a higher contribution of the CD14$^+$ CD16$^+$ subpopulation within the total monocytes (Supplementary Figure S1).

Modulation of chemokine receptors and activation markers on myeloid DC during treatment

To assess whether the decline in the frequency of BDCA-1$^+$ myeloid DC during therapy may be due to altered migration, we examined the expression of chemokine receptors and adhesion molecules on myeloid DC (Supplementary Figure S2). As shown in Figure 2A, the expression of the homeostatic chemokine receptor CCR7 was not altered as a consequence of therapy, neither was the expression of the adhesion molecule CD11a. For both therapy groups, a trend was observed for augmented expression of the inflammatory chemokine receptors CCR4 and CCR5 on myeloid DC following start of treatment. However, statistically only the peginterferon therapy group showed a significant increase at 24 weeks during the

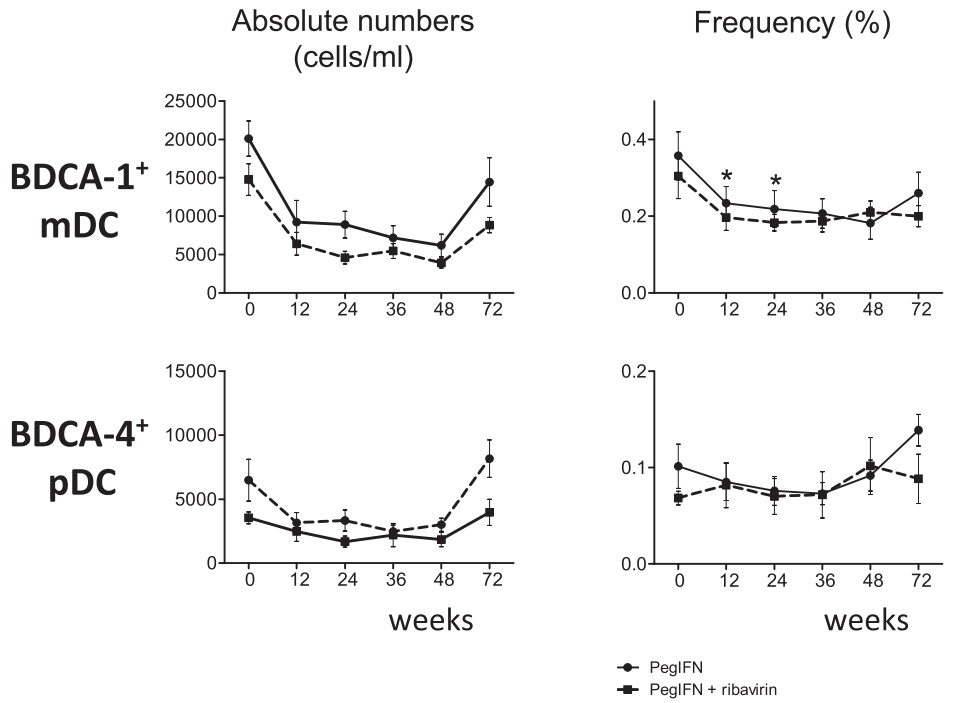

Figure 1. Frequency of antigen presenting cells during IFN-based therapy in chronic HBV patients. Frequencies of myeloid and plasmacytoid DC were determined in whole blood samples by flow cytometry. Myeloid DC were defined as $CD20^-$ $BDCA1^+$ and plasmacytoid DC as $CD123^+$ $BDCA4^+$ cells. Shown are the absolute numbers and frequencies (mean ± SEM) of myeloid DC and plasmacytoid DC within total PBMC from patients treated with peginterferon alone (n=8) or with the combination of peginterferon and ribavirin (n=6). *p<0.05 compared to t=0, Wilcoxon signed-rank test.

treatment period. Besides the effect on chemokine levels, we also analyzed the modulation of the activation status of myeloid DC as a consequence of therapy and the addition of ribavirin (Supplementary Figure S2). During therapy, the basal expression of the co-stimulatory molecules CD40, CD80 and CD86 on myeloid DC showed a mild upregulation, especially early after start of therapy, whereas HLA-DR expression on myeloid DC was not affected (Figure 2B). Significant therapy-induced enhanced expression of costimulatory molecules on myeloid DC were observed in the group receiving peginterferon plus ribavirin, but not in the group receiving peginterferon alone.

Peginterferon with or without ribavirin therapy does not lead to augmented T cell proliferation or T_H1 development

To investigate whether monotherapy or combination therapy differently affects T cell activity, we determined T-cell proliferation and differentiation using patient-derived PBMC. As shown in Figure 3A, HBcAg-specific T-cell proliferation declined shortly after start of treatment, remaining low during the entire treatment phase, and increasing again in a small number of patients after termination of therapy (at week 72; Figure 3A top panel). Although CMV-specific T-cell proliferation was higher than

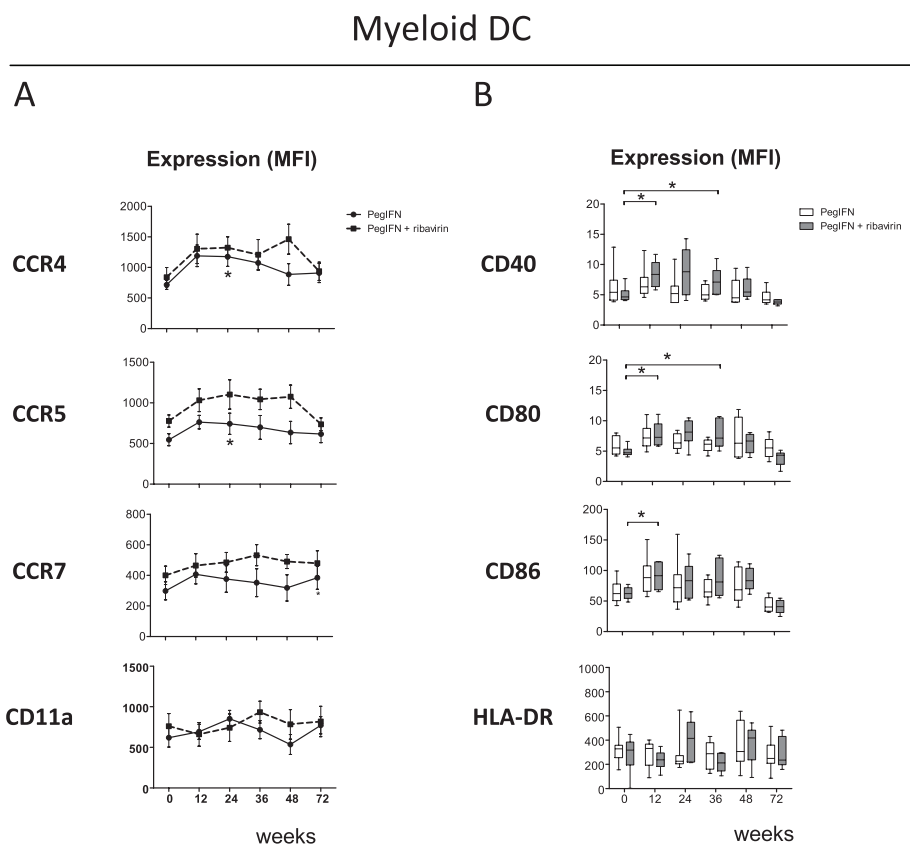

Figure 2. Therapy increases basal maturation status of myeloid DC. The expression levels of chemokine receptors, adhesion molecules and co-stimulatory molecules on myeloid DC was determined by flow cytometry in PBMC from patients treated with peginterferon alone (n=8) or with the combination of peginterferon and ribavirin (n=6). Myeloid DC were defined as $CD3^-$ $CD20^-$ $CD11c^+$ $BDCA\text{-}1^+$. (**A**) Shown are the mean ± SEM expression in MFI of chemokine receptors CCR4 and CCR5 (inflammatory chemokine receptors), CCR7 (homeostatic chemokine receptor) and CD11a. *p<0.05 compared to t=0, Wilcoxon signed-rank test. (**B**) Shown are boxplots with whiskers from minimum to maximum of the expression of maturation markers CD40, CD80, CD86 and HLA-DR ex vivo. *p<0.05 compared to t=0, Wilcoxon signed-rank test.

HBcAg-specific T-cell proliferation, a similar pattern was observed during therapy (Figure 3A middle panel). Polyclonal stimulation of PBMC using antibodies against CD3 showed that during therapy, T cells exhibit less proliferation, but are still viable (Figure 3A bottom panel). The addition of ribavirin to the treatment regimen did not lead to differential T cell proliferative responses as compared to monotherapy.

It has been proposed that interferon-alpha and ribavirin both promote the development of T_H1 cells or lead to higher levels of IFNγ *in vitro* [11-13, 19, 20]. To determine whether addition of ribavirin to the treatment regimen consisting of peginterferon leads to more potent T_H1 cell development, we evaluated the T-helper cell profile as defined by their cytokine production during the course of therapy (Supplementary Figure S3). Upon polyclonal stimulation of PBMC, the percentage of IFNγ-producing T cells decreased during therapy, whereas no effect of treatment was observed for the frequency of IL-4-producing T cells (Figure 3B). Importantly, there were no differences

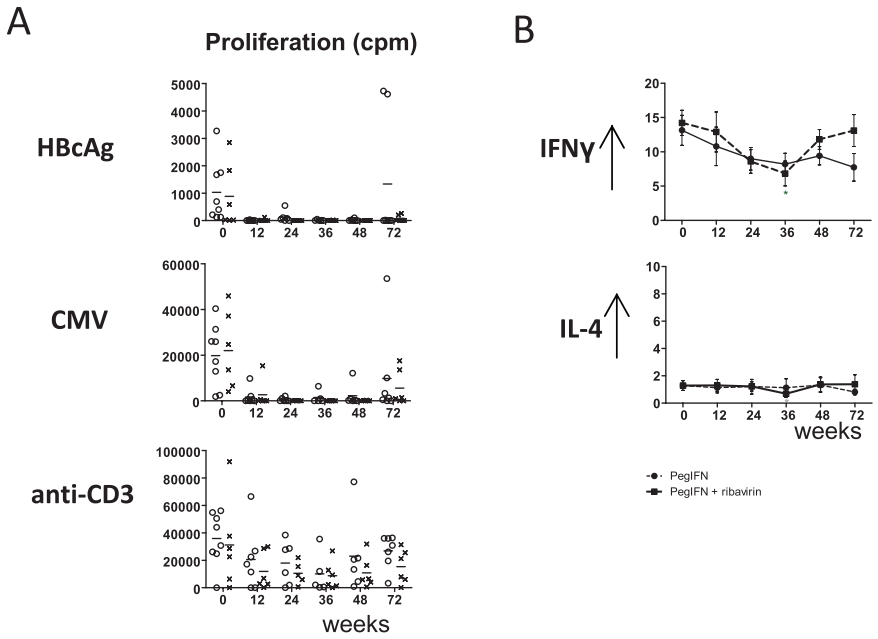

Figure 3. Addition of ribavirin to peginterferon therapy neither increases proliferation nor induces skewing towards a T_H1 profile. (**A**) PBMC from patients treated with peginterferon alone (n=8) or with the combination of peginterferon and ribavirin (n=6) were stimulated with HBcAg (top panel), CMV (middle panel) or anti-CD3 (bottom panel). Depicted is the proliferation in cpm of an individual patient, open circles (peginterferon therapy) or crosses (peginterferon and ribavirin therapy). Lines reflect the grand mean of proliferation within a therapy group for that given time point. (**B**) PBMC were stimulated with PMA and ionomycin and analysed for IFNγ and IL-4 production by intracellular cytokine staining gated on $CD4^+$ T cells. Cytokine production is presented as mean ± SEM IFNγ-positive or IL-4-positive T_H cells. *p<0.05 compared to t=0, Wilcoxon signed-rank test.

between the two therapy groups, indicating that T-helper cell differentiation was not changed towards a more pronounced T_H1 profile as a consequence of treatment with peginterferon or peginterferon and ribavirin combination therapy in chronic HBV patients.

Combination therapy with ribavirin leads to stronger and more sustained plasmacytoid DC activation

As with myeloid DC, we also checked for chemokine expression levels on plasmacytoid DC (Supplementary Figure S4). In accordance with what was seen on myeloid DC, expression of the inflammatory chemokine receptors CCR4 and CCR5 increased on plasmacytoid DC regardless of the therapy group (Figure 4A). Interestingly, expression of the homeostatic chemokine receptor CCR7 was increased in both groups, but was most pronounced in patients treated with the combination of peginterferon and ribavirin, albeit not significantly. No significant differences were observed for CD11a on plasmacytoid DC during the course of treatment (Figure 4A). Next, we investigated whether the activation status of plasmacytoid DC was affected by IFN-based treatment in combination with ribavirin. Compared to a transient effect of peginterferon alone, combination therapy induced a higher upregulation of basal

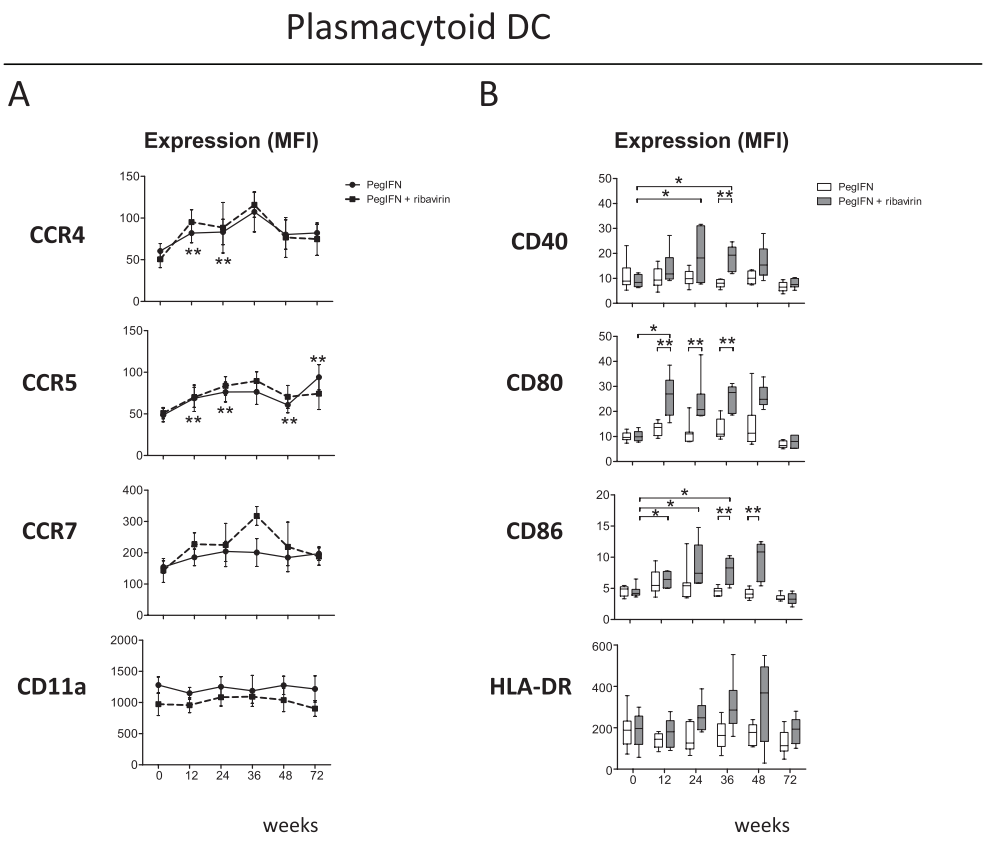

Figure 4. Addition of ribavirin to peginterferon therapy enhances and prolongs augmentation of plasmacytoid DC basal maturation status. Chemokine receptor, adhesion molecules and maturation marker expression by plasmacytoid DC was assessed in PBMC by flow cytometry. plasmacytoid DC were defined as $CD3^-$ $CD20^-$ $CD11c^-$ $BDCA\text{-}4^+$. (**A**) Shown are the mean ± SEM expression in MFI of chemokine receptors CCR4 and CCR5 (inflammatory chemokine receptors), CCR7 (homeostatic chemokine receptor) and CD11a. *p<0.05 compared to t=0, Wilcoxon signed-rank test. (**B**) Shown are box plots with whiskers from minimum to maximum of the expression of co-stimulatory markers CD40, CD80, CD86 and MHC class II molecule HLA-DR ex vivo. *p<0.05 compared to t=0, Wilcoxon signed-rank test. **p<0.05 compared between therapy groups, Mann–Whitney U test.

CD40, CD80, CD86 and HLA-DR expression on plasmacytoid DC that was sustained during the entire treatment period (Figure 4B).

Ribavirin does not influence IFNα production by plasmacytoid DC

It was previously reported that plasmacytoid DC with a more mature phenotype produce less IFNα, a key cytokine produced by plasmacytoid DC [21]. We therefore examined whether the addition of ribavirin to peginterferon monotherapy led to modulation of IFNα levels upon stimulation with the TLR9-ligand CpG. A decline of

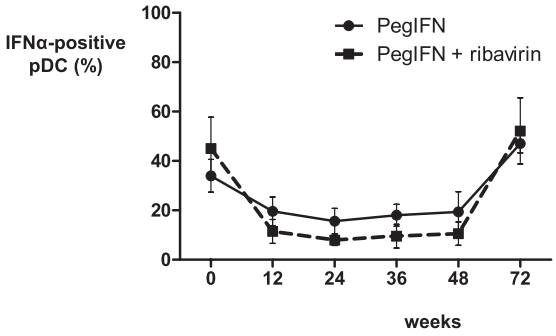

Figure 5. IFNα production by plasmacytoid DC upon CpG stimulation is not influenced by addition of ribavirin to peginterferon monotherapy. Percentage of IFNα-producing plasmacytoid DC in PBMC upon CpG stimulation. PBMC were obtained from patients treated with peginterferon alone (n=8) or with the combination of peginterferon and ribavirin (n=6) and were defined as $CD3^-$ $CD20^-$ $CD123^+$ BDCA-4^+.

IFNα-positive plasmacytoid DC was observed during therapy, and this was observed for both therapy regimens to the same extent (Figure 5). Thus, the addition of ribavirin did not alter the percentage of IFNα-positive plasmacytoid DC as compared to peginterferon monotherapy.

Discussion

To better understand the underlying mechanisms of the nucleoside analogue ribavirin in antiviral therapy, we investigated whether the addition of ribavirin to peginterferon monotherapy had any effect on the numbers, phenotype and function of PBMC derived from chronic HBV patients. We observed that the addition of ribavirin to the IFN-based therapy had no effect on the frequencies of antigen presenting cells. Also no differences were observed on the expression levels of chemokine receptors, and T cell activity was not different between both treatment groups. However, the basal activation status of myeloid and plasmacytoid DC was higher on cells from HBV patients receiving peginterferon and ribavirin combination treatment as compared to monotherapy. As we previously reported, the addition of ribavirin to peginterferon did not improve treatment outcome compared to peginterferon monotherapy in chronic HBV patients [6]. This lack of clinical effect of ribavirin in HBV patients allowed us to assess the immunological effects of adding ribavirin independently of a simultaneous decrease in HBV-DNA.

Myeloid DC are crucial players in antiviral immune responses [22]. We therefore first examined the effect of ribavirin on myeloid DC function, and show that the addition of ribavirin to peginterferon monotherapy did not affect the number and phenotype of myeloid DC in chronic HBV patients. The expression of costimulatory molecules was weakly higher in myeloid DC obtained from patients exposed to ribavirin. These findings differ from the observations in other models, including *in vitro* cell-culture systems [15, 16]. The mildly enhanced myeloid DC activation by ribavirin was not reflected by the degree of HBcAg-specific and CMV-specific proliferation, which was weak, and could not be improved by the addition of ribavirin.

Ribavirin has been suggested to mount a T_H1 type immune response by promoting the production of T_H1-inducing cytokines – such as IL-12 – by DC [16]. Some researchers have shown that ribavirin can skew towards a T_H1 subset phenotype and increase IFNγ production, also during HBV infection [11-13]. Although T_H1 induction is known to be crucial in antiviral immunity [22, 23], we found that ribavirin does not promote T_H1 activity. This apparent discrepancy may well be explained by differences in experimental setup. Unlike these earlier studies, which examined the effect of ribavirin in *in vitro* models [11-13] or an *in vivo* mouse model [11], we use clinical samples derived from chronic HBV patients treated with ribavirin. Aside from a T_H1 effect, we also checked ribavirin for a possible T_H17-promoting effect; however, due to low and often undetectable levels of IL-17 production in our cultures, we could not conclude that ribavirin modulates this response (data not shown).

Analysis of *ex vivo* obtained plasmacytoid DC showed that basal expression of costimulatory molecules was significantly higher during ribavirin and peginterferon combination therapy than during peginterferon monotherapy, albeit that these plasmacytoid DC still exhibited an immature phenotype. From these *in vivo* observations we can not draw firm conclusions about the functional consequences of enhanced expression of CD40, CD80, and CD86. However, it is likely that stronger expression of costimulatory molecules may affect the quality of the T cell response. Alternatively, interactions of plasmacytoid DC with non-T cells such as NK cells may be modulated by a more activated phenotype of plasmacytoid DC. Furthermore, it is important to note that increased maturation of plasmacytoid DC is accompanied with a decreased capacity to produce IFNα [21], which may suggest a shift of plasmacytoid DC function towards cellular immune responses. Although we show that IFNα production by plasmacytoid DC upon CpG stimulation during therapy is reduced, no differences in IFNα production between peginterferon and peginterferon and ribavirin therapy were observed. This suggests that administration of peginterferon, rather that ribavirin, leads to suppression of IFNα production by plasmacytoid DC, and is not caused by an altered activation status of plasmacytoid DC. Recently, levels of CD80 on plasmacytoid DC of chronic HCV patients were found to be predictive for treatment outcome [24], further demonstrating the functional importance of differential plasmacytoid DC activation.

Since ribavirin is a purine analogue, it is possible that it may directly trigger TLR7- or TLR9-mediated signalling, and consequently activate plasmacytoid DC. Although, on the basis of our findings, we can not conclude whether plasmacytoid DC were activated directly or indirectly upon administration of ribavirin, others have shown previously that TLR7 transfected cells are only minimally activated by ribavirin at supra-physiological concentrations *in vitro*, and no activation by TLR9 transfected cells [25].

In conclusion, our findings demonstrate that addition of ribavirin to interferon-based therapy of chronic HBV patients increases, although mildly, the activation status of myeloid and plasmacytoid DC during treatment. Further studies are needed to examine whether the effects of ribavirin on the DC compartment in chronic HBV patients are disease specific, and thus whether these effects may explain why the addition of ribavirin to peginterferon monotherapy was not clinically effective in chronic HBV patients.

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Disclosures

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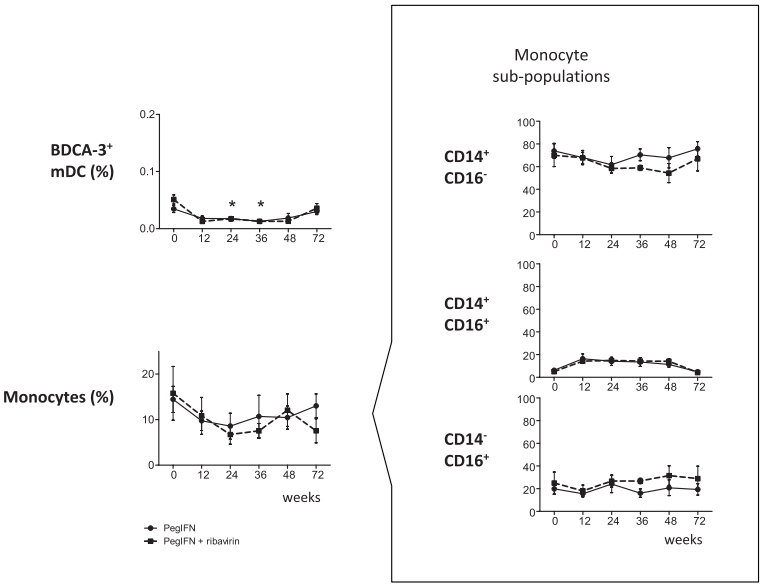
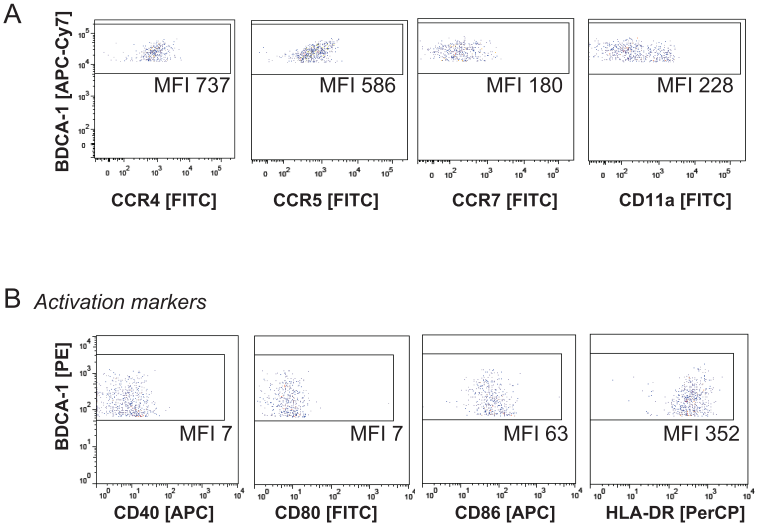
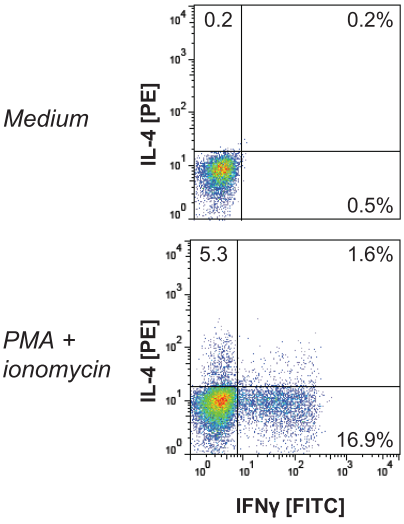
Data supplement



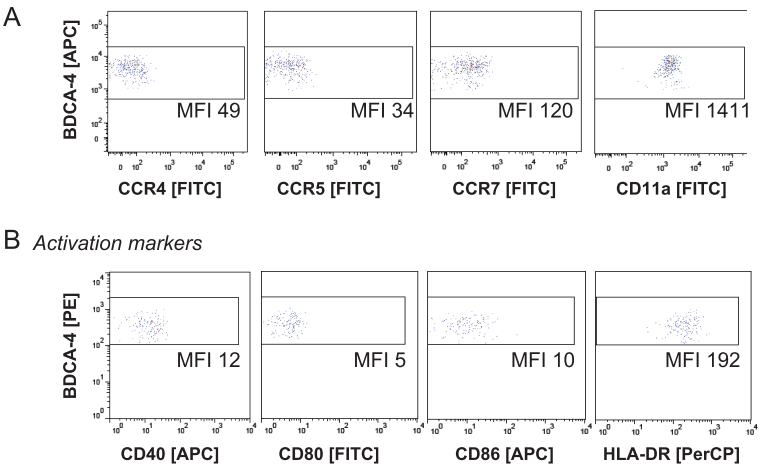
Supplementary figure 1. Frequency of BDCA-3$^+$ myeloid DC and monocytes during IFN-based therapy in chronic HBV patients. Frequencies of BDCA-3$^+$ myeloid DC and monocytes were determined in whole blood samples by flow cytometry. Monocytes are defined as CD3$^-$ CD20$^-$ CD56$^-$ BDCA-1$^-$ BDCA-4$^-$ and CD14$^+$ and/or CD16$^-$. Shown are the mean ± SEM percentages of DC and monocytes within total PBMC, and mean ± SEM percentages of monocyte subsets (CD14$^+$ CD16$^-$, CD14$^+$ CD16$^+$, CD14$^-$ CD16$^+$) within total monocytes derived from patients treated with peginterferon alone (n=8) or with the combination of peginterferon and ribavirin (n=6). *p<0.05 compared to t=0, Wilcoxon signed-rank test.



Supplementary figure 2. Analysis of BDCA-1$^+$ myeloid DC phenotype by flow cytometry. Representative pseudocolor density plots of chemokine receptor expression levels (**A**) and costimulatory molecule levels (**B**) on myeloid DC at baseline; expression levels depicted as geometric mean fluorescence intensity (MFI).



Supplementary figure 3. Analysis of T helper cell cytokine response by flow cytometry. Representative pseudocolor density plots showing IFNγ-producing and/or IL-4-producing T helper cells upon stimulation of PBMC at baseline with medium or PMA-ionomycin; cytokine-producing cells depicted as percentage of total $CD4^+$ T helper cells.



Supplementary figure 4. Analysis of plasmacytoid DC phenotype by flow cytometry. Representative pseudocolor density plots of chemokine receptor expression levels (**A**) and costimulatory molecule levels (**B**) on plasmacytoid DC at baseline; expression levels depicted as geometric mean fluorescence intensity (MFI).

IFNλ-mediated IL-12 production in macrophages induces IFNγ production in human NK cells

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Chapter 4

Abstract

With increasing interest in alternative options to interferon-alpha (IFNα)-based treatments, IFNλ has shown therapeutic promise in a variety of diseases. Although the antiviral activity of IFNλ has been extensively studied, there is limited knowledge regarding the immunological functions of IFNλ and how these differ from those of other classes of IFNs. In this study, we investigated the effects of IFNλ on natural killer (NK) cells, both in a direct and indirect capacity. We demonstrate that in contrast to IFNα, IFNλ is unable to directly stimulate NK cells, due to the absence of IFNλ receptor chain 1 (IFNλR1) on NK cells. However, IFNλ, 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, IFNλ is able to indirectly affect NK cells and ultimately induce IFNγ 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 (IFNγ) 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 cell activation ensues after interacting with altered 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 [1-3]. 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 [4-7]. 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 (IFNα), e.g. during interaction with IFN-producing activated plasmacytoid DCs leading to enhanced activation and cytolytic activity [8-10], and thereby promoting antiviral immunity.

In recent years the type III family of IFNs, comprised of IFNλ1, IFNλ2, IFNλ3, and IFNλ4, 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) [11-14]. All members of the IFNλ family use a specific receptor heterodimer, the IFNλR1 and IL-10R2 complex, which structurally differs from the IFNα receptor complex, but is similar in its triggering of downstream JAK/STAT signal transduction, ultimately resulting in the initiation of gene transcription. In contrast to the ubiquitously expressed IFNα receptor, the distribution of the IFNλ receptor is more limited. Murine models have shown that epithelial cells express the receptor complex and responded to IFNλ stimulation by eliciting an antiviral response [15]. Outside of non-hematopoietic cells, specifically epithelial cells and hepatocytes, expression of the IFNλR1 chain in humans has been well-described on plasmacytoid DCs, fibroblasts [16, 17], and recently on macrophages [18].

We have previously demonstrated that monocyte-derived macrophages expressed a functional IFNλ 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. This was in contrast to IFNα, which inhibited TLR-induced IL-12p40 production in monocyte-derived macrophages. Further functional differences have been described between IFNα and IFNλ in their modulation of hepatocytes, where IFNλ has been shown to activate a more restricted subset of cells as well as induce weaker interferon-stimulated gene responses [19, 20].

To further define a role for IFNλ in antiviral immune responses, the goal of this study was to determine whether IFNλ 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 IFNλ as being able to partially inhibit NK cell-derived IFNγ production upon IL-12/15 stimulation [21, 22], but an effect of IFNλ on NK cells could not be reproduced in an ensuing response to the original article [23]. To better understand the biological role of IFNλ, we investigated the effects of IFNλ on NK cells in both a direct and indirect capacity through its modulation of TLR-activated macrophages.

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 $0.75*10^6$ cells/ml in RPMI1640 medium (Lonza) supplemented with 10% fetal calf serum (Sigma), penicillin/streptomycin (Gibco), L-glutamin (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 IFNλ 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 IFNα-2b (Intron A, Merck) or 100 ng/ml IFNλ1 (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 $1x10^6$ 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 IFNγ (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 IFNα-2b (Intron A, Merck) or 100 ng/ml IFNλ1 (Bristol-Myers Squibb) alone or in combination with 100ng/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 IFNαR1/2 (MRHAR-2, Millipore) and IL-12p40 (C8.6, 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-IFNγ-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 Incorperated).

NK cell CD107a degranulation

Isolated NK cells ($0.1x10^6$ cells/200 µl) were seeded with K562 cells in a 96 well plate in a 1:1 effector to target ratio. IFNα-2b (10 ng/ml; Intron A, Merck) or recombinant IFNλ1 (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.

Quantification of IFNαR1/2, IFNλR1, and IL-12 family subunits mRNA expression

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), IFNαR1 (Hs01066115_m1), IFNαR2 (Hs01022060_m1), IFNλR1 (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 are 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.

Results

IFNαR1/2 is expressed across primary NK cells, monocytes, and NK92/THP-1 cell lines, whereas IFNλR1 is only expressed after monocyte to macrophage differentiation

The direct effects of IFNα on NK cells have been well described [9, 10], but the ability of IFNλ 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 IFNλ receptor. This was achieved by comparing the mRNA expression of IFNα and IFNλ receptors on primary NK cells and NK92 cells by quantitative PCR (qPCR). A HEK293 cell line stably transfected to express IFNλ receptor mRNA as well as primary human hepatocytes, known to express the receptor and respond to IFNλ [19, 20], 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 IFNαR1 and IFNαR2 chains were ubiquitously expressed across all primary cells and cell lines (Figure 1A), IFNλR1 mRNA expression was not detected in either primary NK or NK92 cells (Figure 1B). Monocytes were also devoid of IFNλR1 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 IFNλR1 over a 6 day period (Figure 1C). IFNαR1 and IFNαR2, both already highly expressed on monocytes, remained relatively unaltered across the differentiation process.

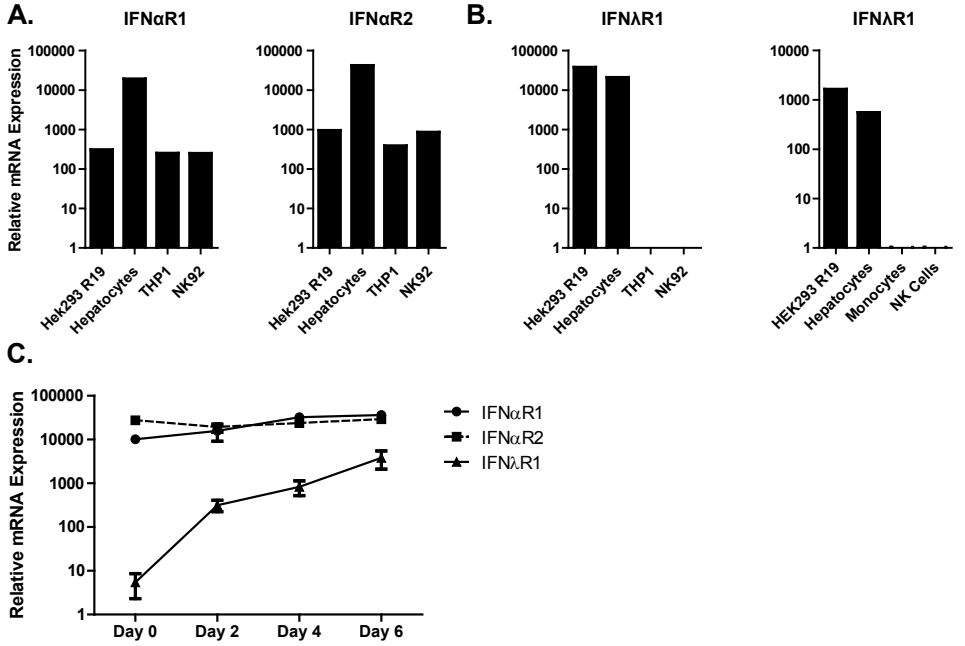

Figure 1. IFNαR1/2 is expressed across primary NK cells, monocytes, and NK92/THP-1 cell lines, whereas IFNλR1 is only expressed after monocyte to macrophage differentiation. qPCR assays for IFNαR1, IFNαR2 (both **A**), and IFNλR1 (**B**) were performed on THP-1 and NK92 cell lines as well as the primary NK cells and monocytes, using primary hepatocytes and HEK293 cells stably transfected to express IFNλR1 as positive controls. (**C**) mRNA expression of IFNαR1, IFNαR2, and IFNλR1 measured in monocytes (n=6) at day 0, 2, 4, and 6 of culture with GM-CSF to induce macrophage differentiation.

IFNα, but not IFNλ1, 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 IFNα or IFNλ1 overnight and analyzed for their expression of various activation markers and inhibition/activation receptors, including CD69, an 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 seven-fold in IFNα cultures (76.3%) compared to those exposed to medium alone (11.3%) or IFNλ1 (13.5%) (Figure 2A) while expression of inhibitory receptor NKG2A remained unaffected in all conditions. The cytolytic capacity of IFN-

stimulated NK cells was assessed by measuring the degranulation marker CD107a after co-culture with the target cell line K562, and by mRNA expression of protease granzyme B (GZMB). NK cells exposed to IFNα during the 5 hour co-culture period with K562 cells expressed higher levels of CD107a (32.2%) while co-culture with IFNλ1 (21.1%) showed no difference from the medium condition (19.8%) (Figure 2B). Stimulation with IFNα, in combination with IL-18, was also able to upregulate GZMB expression 10-fold after 5 hours, whereas IFNλ1 again remained ineffective (Figure 2C). Lastly, isolated primary NK cells were incubated with either IFNα or IFNλ1, in addition to IL-18, overnight and analyzed by intracellular flowcytometry for production of IFNγ and TNF (Figure 2D). In contrast to IFNα, IFNλ1 was unable to directly increase the frequency of primary NK cells producing IFNγ upon IL-18 stimulation. This highlights a direct action of IFNα on NK cells, resulting in activation, cytotoxicity, and cytokine production, that does not exist for IFNλs, most likely due to the absence of IFNλ receptor expression.

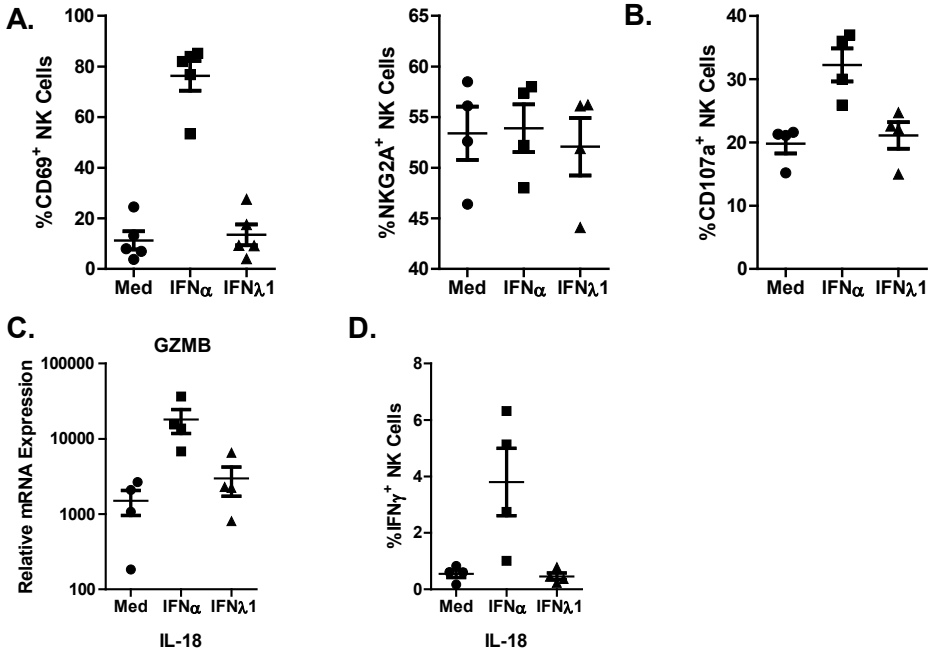

Figure 2. IFNα, but not IFNλ1, directly stimulates and enhances effector function in NK cells. (**A, D**) Surface marker and intracellular FACS analysis of CD69 (n=5), NGK2A (n=4), IFNγ (n=4) in isolated cells that were stimulated with IFNα or IFNλ1 alone or in combination with IL-18 overnight. IFNα was able to induce NK cell activation and enhance IL-18 induced IFNγ in NK cells, whereas IFNλ1 had no direct effect. (**B, C**) The cytolytic capacity of IFN stimulated NK cells was assessed by measuring degranulation marker CD107a after co-culture with target cell line K562 (n=4), and by qPCR to measure mRNA expression of protease granzyme B (GZMB) (n=4). Elevated levels of both CD107a and GZMB expression were only seen in the IFNα condition.

TLR-mediated IL-12 family cytokine expression is differentially modulated by IFNλ1 and IFNα 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 [4-7]. Although we determined that IFNλ1 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 IFNα or IFNλ1, were further challenged with TLR4 agonist LPS for an additional 4 hours for gene expression analysis or 24 hours for immunoassay analysis of pro-inflammatory cytokines. To determine to what extent IFNλ1 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 was observed in IFNλ1/LPS-stimulated macrophages compared to the IFNα/LPS conditions. IL27A (IL-27p28) mRNA was upregulated by incubation of both IFNα/LPS and IFNλ1/LPS, whereas IL12A (IL-12p35) mRNA expression was undetectable in all conditions (data not shown).

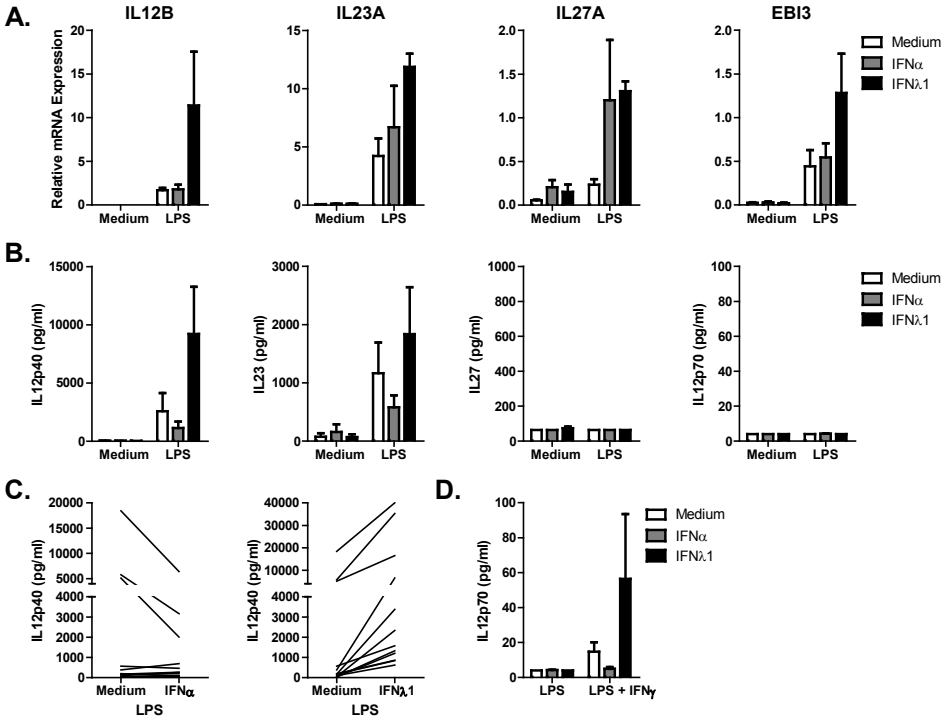

Figure 3. TLR-mediated IL-12 family of cytokines are differentially modulated by IFNλ1 and IFNα in macrophages. (**A**) mRNA (n=3) and (**B**, **C**) protein (n=13) levels of the IL-12 family members in macrophages stimulated with IFNα or IFNλ1 for 5 hours and then further challenged with LPS for an additional 4 or 24 hours, respectively. IFNλ induced upregulation of IL12B, IL23A, IL27A, and EBI3 mRNA expression, but only IL-12p40 and IL-23 were detected at a protein level in the supernatants of these activated macrophages. (**D**) The addition of IFNγ to LPS stimulation of macrophages resulted in the production of bioactive IL-12p70 (n=8).

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 IFNλ1/LPS-stimulated cells, in contrast to IFNα/LPS stimulation (Figure 3B-C). IL-12p70 and IL-27 were not detected in any condition, partially explained by the lack IL-12p35 mRNA expression previously seen by qPCR.

Bioactive IL-12 was produced by supplementation of IFNγ to the TLR challenge of macrophages after the initial priming with IFNλ1, but not after IFNα pre-treatment (Figure 3D). Overall, differences between IFNα and IFNλ1 in modulating TLR induced production by macrophages were observed across multiple members of the IL-12 family.

IFNλ1-mediated IL-12 production by macrophages induces IFNγ production by NK and NK92 cells

To test the effects of IFN on cross-talk between macrophages and NK/NK92 cells, supernatants from macrophages incubated with IFNα or IFNλ1 in combination with LPS and IFNγ 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 IFNγ and TNF production in the respective conditions. Supernatants from IFNλ1/LPS-stimulated macrophages were able to increase the percentage of IFNγ-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, B). The same was true for NK92 cell cultures, where a 2.5-fold increase in IFNγ positive cells was observed compared to NK92 cell

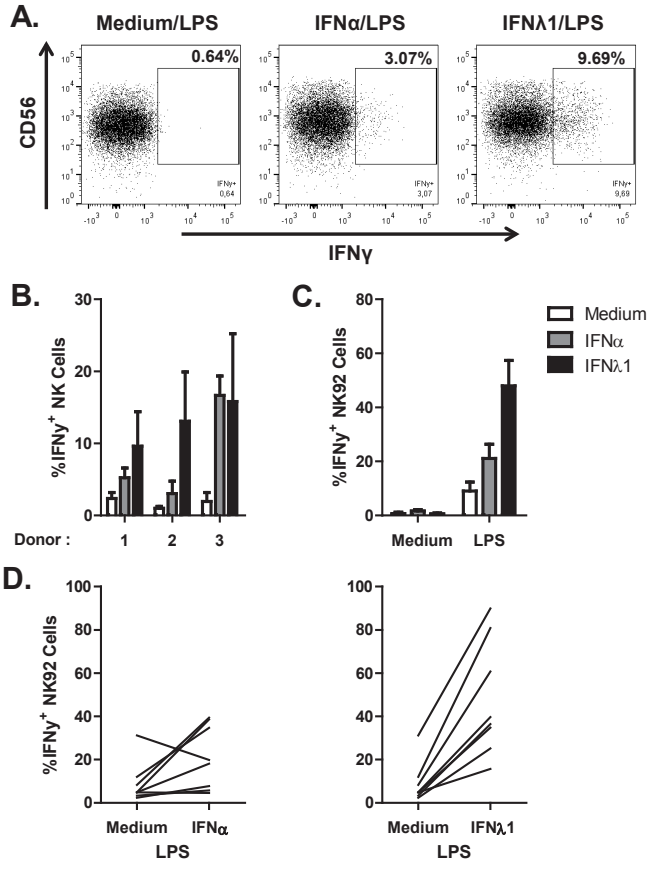

Figure 4. IFNλ1 mediated IL-12 production of macrophages is able to induce effector function in NK and NK92 cells. (A-C) Supernatant from macrophages treated with IFNα or IFNλ1 in combination with LPS and IFNγ were used to stimulate isolated primary NK cells from 3 donors (n=5 per donor) and NK92 (n=8) cells overnight supplimented with IL-18. Supernatants from macrophages treated with IFNλ1 induced a significantly higher frequency of IFNγ-producing NK cells compared to the medium control, and NK92 cells showed an increase in IFNγ-producing cells compared to both the medium control and IFNα conditions. (D) Individual results for supernatants of multiple donors for macrophage-mediated IFNγ production in NK92 cells.

cultures treated with supernatants from both medium/LPS and IFNα/LPS stimulated macrophages (p=0.008) (Figure 4C, D). It should be noted that increased IFNγ production was also seen with the IFNα pre-treated supernatants; this can partially be attributed to the direct action of IFNα on NK and NK92 cells.

IFNγ production in NK92 cells is IL-12 dependent in IFNλ1/LPS-stimulated but not in IFNα/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 IFNλ1/LPS-stimulated macrophages abrogated IL-12-mediated IFN production in NK92 cells (p=0.01) while neutralization of IL-12p40 in IFNα/LPS-stimulated macrophages partially abrogated IL-12-mediated IFN production in NK92 cells (p=0.01) (Figure 5A-C). Blocking of the IFNα receptor in macrophages stimulated with IFNα/LPS showed a strong, but not significant, trend in reduction in IFNγ production in NK92 cells(p=0.065), however no effect was observed after stimulation with IFNλ1/LPS. These results confirm the direct action of IFNα on NK cell effector function, and demonstrate that IFNλ1 acts indirectly on NK cells through macrophage-derivedIL-12 production.

Discussion

The focus of this study was to investigate the ability of IFNλ1 to modulate the activity of NK cells in both a direct and an indirect capacity. We demonstrate that unlike IFNα, IFNλ1 is unable to directly activate and promote effector function in NK cells, presumably due to the lack of cellular expression of the type III IFN receptor complex. However, IFNλ1 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 IFNλ1 is able to indirectly affect NK cells through macrophage-mediated IL-12 production, ultimately inducing IFNγ production.

As a first step, we demonstrate that NK cells do not express the IFNλR1 chain, and that consequently IFNλ1 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 IFNγ production upon IL-12/IL-15 stimulation [21, 22]. This 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 IFNα is able to activate as well as promote cytolytic action and IFNγ production by NK cells, whereas IFNλ1 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 IFNλR1 receptor expression in primary NK cells and the inability of IFNλ to induce IFNγ production or degranulation in any capacity [23, 24]. Combined, these results convincingly show that IFNλ 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 IFNλ1 and

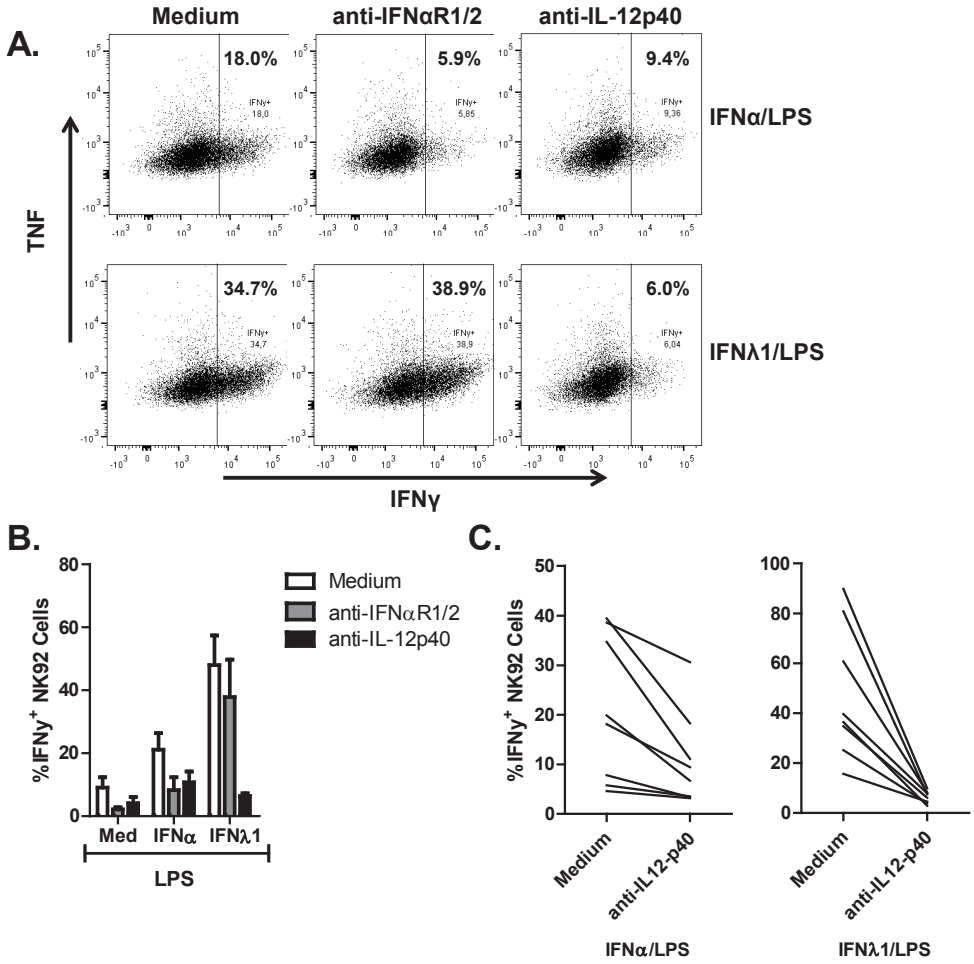

Figure 5. Macrophage-mediated IFNγ production in NK92 cells is IL-12-dependent in IFNλ1/LPS but not in IFNα/LPS-stimulated macrophages. (**A**,**B**) Neutralization of IL-12p40 in IFNλ1/LPS-stimulated macrophages abrogated IL-12-mediated IFNγ production in NK92 cells but only partial reduced IFNγ production after IFNα/LPS stimulation. Blocking of the IFNα receptor reduced the frequency of IFNγ-producing NK92 cells after treatment with supernatants from macrophages stimulated with IFNα/LPS, but had no effect on the IFNλ1/LPS stimulations (n=8). (**C**) Individual results for IL-12p40 neutralization in supernatants from multiple donors for macrophage-mediated IFNγ production in NK92 cells.

NK cells, mediated through the stimulation of macrophages. This interaction was unique to IFNλ1, due to differences in immunomodulatory properties between IFNα and IFNλ1, specifically in their regulation of various subunits of the IL-12 family of cytokines (IL12B, IL23A, EBI3) in macrophages. In contrast to IFNλ1, which promoted the production of specific IL-12-related cytokines, IFNα actually reduced macrophage-facilitated IL-12 production. Through the blocking of IFNαR1/2 on NK cells and neutralizing IL-12 in supernatants from IFN-primed macrophages, we were able to show that enhancement of NK-celled derived IFNγ production by IFNα was mainly mediated by a direct interaction with NK cells, and minimally affected by IL-12 neutralization. IFNλ1, on the other hand, was dependent on IL-12, with almost complete abrogation of IFNγ-producing NK cells after IL-12 neutralization. Despite

having different modes of action, both the direct effects of IFNα and indirect effects of IFNλ1 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 IFNγ production in NK cells. 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 IFNγ and GM-CSF in NK cells [25-27]. Although the production of IL-23 and IL-27 was monitored in IFNλ-stimulated macrophages, the effects on NK cell function was not explored in this study.

In contrast to the ubiquitous expression of the IFNαR1/2 subunits across various cell populations, resting monocytes do not express IFNλR1 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 IFNα receptor on NK cells and the ability of IFNα to directly activate these cells, promoting degranulation, cytotoxicity, and IFNγ production, is distinct from the effects of IFNλ. These features of IFNα 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 IFNRλ1 prevented a direct action of IFNλ on both NK cells and monocytes, with only responses observed on differentiated macrophages. This suggests that IFNλ might play a more prominent role in tissue-specific responses as compared to the periphery, in line with other studies describing that IFNλ1 has a more delayed and prolonged effect through activation of specific tissue-resident cells, specifically epithelial cells and hepatocytes [19, 20]. TLR-dependent crosstalk 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 IFNγ production [28]. The ability of IFNλ1 to modulate TLR-induced crosstalk between macrophages and NK cells could have implications in the enhancement of IFNγ production by liver NK cells, a critical factor in the inhibition of HCV replication and in the response to HBV infection [29-31]. The delayed activation of NK cells by IFNλ, 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 [20, 24, 32].

Collectively, this study provides the first example of an indirect regulatory effect unique to IFNλ1, mediated through macrophage cytokine production and independent of cell-to-cell contact. This is distinct from IFNα, which directly activates NK cells, and can inhibit macrophage IL-12 production. Further studies of both the antiviral and immunoregulatory effects of IFNλ1 are warranted to understand the basic biology of type III IFNs and their activities as therapeutic agents.

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Authorship Contributions

RdG designed research, performed research, analyzed data and wrote the manuscript; AB designed research, performed research, and analyzed data, JH performed microarray data analysis, BL, FM, JF and HLAJ designed research and wrote the manuscript; AB designed research, analyzed data and wrote the manuscript.

Disclosure of Conflicts of Interest

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

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Monocytes from chronic HBV patients react *in vitro* to HBsAg and TLR by producing cytokines irrespective of stage of disease

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Chapter 5

Abstract

Individuals who are chronically infected with the hepatitis B virus (HBV) are highly heterogenous with respect to serum levels of HBV DNA, HBV particles and viral proteins. Since circulating leukocytes, such as monocytes, are constantly exposed to these viral components, it is likely that the functionality of these cells is affected. However, at present, little information is available on the consequences of the interaction between monocytes and viral components. Therefore, we examined the *in vitro* effects of HBV surface antigen (HBsAg) on monocytes and evaluated whether these effects were reflected *in vivo*.

We observed that *in vitro* HBsAg exposure of monocytes induced robust production of IL-6 and TNF. However, between chronic HBV patients with distinct levels of serum HBsAg, HBV early antigen (HBeAg), and HBV DNA, TLR-induced monocyte cytokine production did not differ. Importantly, HBsAg-induced cytokine production by monocytes was similar between patients and healthy controls showing that earlier *in vivo* exposure to HBsAg does not affect the *in vitro* response. Additionally, we show that IL-10 is able to inhibit cytokine production by HBsAg-induced monocytes.

In conclusion, we demonstrate that monocytes can recognize and respond to HBsAg, resulting in vigorous pro-inflammatory cytokine production *in vitro*. However, phenotype and function of the monocyte compartment in chronic HBV patients are not influenced by differences in levels of serum viral components, suggesting that regulatory mechanisms are active to avoid excessive *in vivo* monocyte activation.

Introduction

Hepatitis B virus (HBV) infection is a major health problem. Although the majority of infected individuals clear the virus spontaneously, a fraction of patients is unable to clear the virus and develops a chronic form of hepatitis. Their numbers have already reached over 240 million people [1]. In time, persistence of HBV can lead to progressive liver damage, which increases the patient's risk of developing liver cirrhosis, liver failure and liver cancer. Chronicity of HBV is the result of a complex interaction between the replicating virus and an inadequate immune response [2-4]. After infection, viral replication takes place inside hepatocytes, and the secretion of infectious virions can take place for decades at high rates, and consequently HBV DNA, as well as viral proteins, like HBV early antigen (HBeAg) and HBV surface antigen (HBsAg), can be easily detected in serum. The levels of these clinical markers may fluctuate over time and are a reflection of disease activity and commonly used to define the patients' disease stage [3,4].

Although circulating monocytes represent about 10% of leukocytes in human blood, relatively little is known on the consequences of chronic viral infections on monocytes. In HIV infections impaired monocyte functions have been reported [5,6], and we recently demonstrated altered Toll-like receptor (TLR) responsiveness of monocytes obtained from patients with chronic HCV infections [7,8]. Monocytes can be divided into two distinct subpopulations that are discerned based on their surface expression of CD14 and CD16. CD14high$CD16^-$ monocytes make up the majority (80-90%) of blood monocytes, and have been reported to produce relatively high IL-10 and weak TNF levels, whereas the $CD14^+CD16^+$ subpopulation produces higher levels of pro-inflammatory cytokines, such as TNF and IL-1β [9,10].

Also in chronic HBV, some studies reported modulation of the monocyte compartment as a result of the disease. Depending on the clinical phase of the chronic HBV infection altered monocyte subsets frequencies were reported [11,12]. Moreover, PBMC from HBeAg-positive patients produced less TNF and IL-6 upon stimulation with TLR2 agonists as compared to HBeAg-negative patients [13], which was explained by lower expression of TLR2 in HBeAg-positive patients [14]. Furthermore, exposure of monocytes to HBsAg suppressed LPS-induced TNF and IL-1β production [15], while others reported that HBsAg has an immunostimulatory effect by inducing TNF and IL-10 production [16]. Since the consequences of constant exposure of peripheral monocytes to viral particles and the viral proteins HBeAg and HBsAg are still not completely understood, we here studied the *in vitro* and *in vivo* effects of these molecules on the phenotype and function of peripheral monocytes.

Materials and methods

Patients and ethics statement

Peripheral blood was collected from patients chronically infected with HBV who visited the outpatient clinic of the Erasmus Medical Center. Patients eligible for the study were positive for HBsAg, and were not on-treatment before blood samples

were taken for this study. Patients co-infected with human immunodeficiency virus, hepatitis A virus, hepatitis C virus or hepatitis D virus were excluded. Patient characteristics are presented in Table 1. The medical ethical committee of the Erasmus MC University Medical Center approved the study and all patients gave written informed consent before inclusion.

Laboratory measurements

HBsAg levels and HBeAg levels were measured in sera from a total of 45 chronic HBV patients using the Architect HBsAg assay (Abbott Laboratories, Abbott Park, IL, USA; range 0.05–250 IU/ml) or HBeAg assay (Abbott Laboratories; interpreted using a ratio of the sample relative light unit (RLU) rate to the cut-off RLU (S/CO)). HBV DNA levels were measured in serum using the Cobas TaqMan (Roche Diagnostics; lower limit of quantification, 20 IU/ml). ALT was measured as part of standard diagnostic procedures. HBV genotype was determined by means of the INNO-LiPA assay (Innogenetics, Gent, Belgium).

Flow cytometric analysis of monocyte subpopulations

To determine the frequencies of monocyte subpopulations, whole blood was lysed and stained with antibodies against CD14 and CD16 (61D3 and 3G8, respectively; both eBioscience, San Diego, CA, USA), and measured by flow cytometry using a BD FACSCanto II (BD Biosciences, San Diego, CA, USA). Data was analyzed using FlowJo 7.6.5 software (Tree Star, Inc., Ashland, OR, USA).

Monocyte stimulation and ELISA

Peripheral blood was collected from chronic HBV patients or healthy controls in sodium-heparin tubes, and PBMC were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) gradient centrifugation, and frozen. PBMC ($1x10^6$ cells/ml) were

Table 1

Patient characteristics

	Group 1	Group 2	Group 3
Number	14	20	11
Sex (F:M)	5:9	11:9	6:5
Age	41.1 (24-59)[a]	35.1 (20-53)	31.4 (18-48)
ALT (IU/L)	83 (21-366)	37 (15-102)	111 (22-443)
Viral load (IU/mL)	$63.8x10^6$ (28,300-$6.0x10^8$)	1,767 (20-6,490)	$1.0x10^9$ (29,200-$2.6x10^9$)
HBeAg level (IU/mL)	7.96 (0-66.65)	0.17 (0-2.38)	944 (389-3,259)
HBsAg level (IU/mL)	10,341 (56-31,660)	3032 (108-17,448)	53,581 (542-125,820)

[a] Values for age, ALT, viral load, HBeAg level and HBsAg level are mean (minimum – maximum value)

thawed and stimulated in 96-well plates in 250 µl X-VIVO culture medium (Lonza, Verviers Sprl, Belgium) containing penicillin/streptomycin (Gibco, Paisley, UK), L-glutamin (Lonza), and HEPES (Lonza) as described previously [7]: the cells were cultured either unstimulated or stimulated with 100 ng/ml Toll-like receptor (TLR) 2 ligand Pam3CSK4, 100 ng/ml TLR4 ligand LPS (both from Invivogen), 1 µg/ml TLR7/8 ligand R848 (Enzo Life Sciences, Antwerp, Belgium), or human plasma-derived pHBsAg Ay (American Research Products (ARP), Waltham, MA, USA) at a concentration of 5 µg/ml unless mentioned otherwise. In the experiment shown in Figure S1, in parallel to plasma-derived HBsAg Ay (5 µg/ml) stimulation, PBMC were also stimulated with recombinant HBsAg (1 µg/ml; Prospec, Rehovot, Israel) for 5 hours. In the IL-10 inhibition experiment, 10 ng/ml IL-10 (Miltenyi Biotec, Bergisch Gladbach, Germany) or medium as a negative control, was added at the same time as pHBsAg (1 µg/ml; ARP). After a total of 18 hours of culture, supernatants were harvested, and cytokine production (IL-6 and TNF) was determined by ELISA (all kits from eBioscience).

Intracellular cytokine staining

The frequency of cytokine-producing monocytes was determined by measuring cytokines with intracellular cytokine staining using flow cytometry. PBMC were stimulated with TLR ligands or pHBsAg Ay (ARP) as described above, After culturing for 2 hours, brefeldin A (10 µg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to all wells. After 16 hours, the cells were harvested, incubated with LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen, Ltd., Paisley, United Kingdom), fixed with 2% formaldehyde, permeabilized with 0.5% saponin (VWR, West Chester, PA, USA) and stained for IL-6 (MQ2-13A5; eBioscience), and TNF (Mab11; eBioscience), and the surface markers CD14 and CD45 (61D3 and HI30, respectively; both eBioscience). Flow cytometric data was acquired and analyzed as described above.

Statistical analysis

To compare clinical groups, groups were analyzed using the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparisons test. The frequencies of cytokine-producing cells determined at different HBsAg doses were normalized to the values obtained under the 1 µg/ml HBsAg condition. Differences between the cytokine expression of the healthy individuals and the group with chronic HBV patients were analyzed using the Mann-Whitney U test. A P-value of <0.05 was considered statistically significant. Graphpad Prism 5 was used for statistical analysis.

Results

HBsAg induces cytokine production by monocytes *in vitro*

To investigate the modulatory effect of HBsAg on the functionality of blood monocytes, PBMC from healthy individuals were cultured in the presence of HBsAg. We compared monocytes, defined in Figure 1A as CD45 and CD14-expressing

cells with their characteristic FSC and SSC profile, after overnight culture with either medium or HBsAg. As shown in Figure 1B and quantified in Figure 1C, the frequency of cytokine-producing monocytes was increased upon exposure to HBsAg *in vitro*. Upon exposure to HBsAg *in vitro*, the mean frequency of IL-6 and TNF-producing monocytes was increased from 2.6% to 61% and from 3.5% to 59%, respectively (Figure 1C). The percentages of monocytes producing IL-12p40, IL-15, and IL-10 were relatively low, while the chemokines CCL4 and CXCL8 were strongly induced (data not shown).

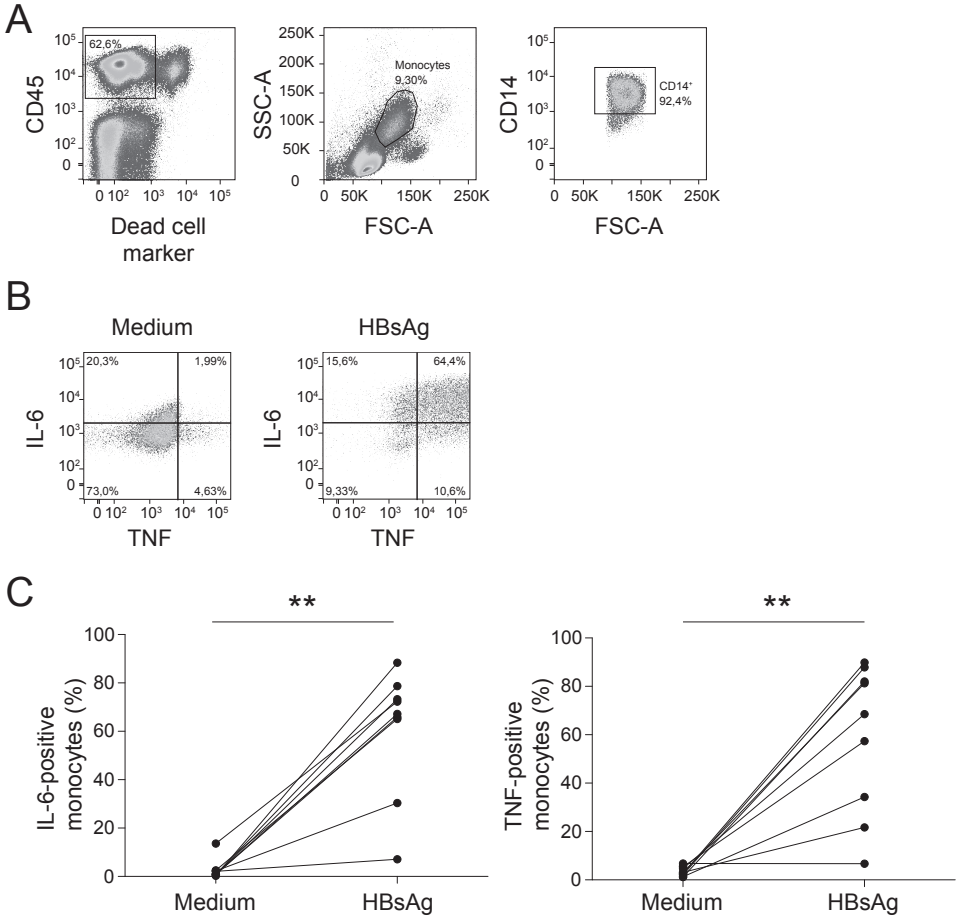

Figure 1. HBsAg induces cytokine production by monocytes. PBMC from healthy individuals were stimulated with HBsAg. After overnight incubation, cells were stained for CD14, IL-6 and TNF. (**A**) Gating strategy: Viable monocytes were identified on the basis of their forward-sideward scatter profile and their membrane expression of CD14. (**B**) Representative intracellular cytokine stainings are presented showing IL-6 and TNF-producing monocytes upon incubation with medium and HBsAg. (**C**) The frequencies of monocytes producing IL-6 or TNF upon incubation with medium or HBsAg are presented (n=9, ** $p<0.01$, Wilcoxon signed rank test).

The ratio of monocyte subpopulations is comparable in chronic HBV patients with different levels of HBV DNA or serum HBV protein levels

The stimulatory effect of HBV proteins on blood monocytes upon exposure *in vitro* may have consequences for the functionality of the monocyte compartment

in patients chronically infected with HBV. To examine this, we selected groups of chronic HBV patients differing in the levels of HBV DNA, HBeAg, and HBsAg. As shown in Figure 2A, three groups were identified based on HBV DNA and HBeAg levels. The experimental groups differed not only in the levels of HBV DNA and HBeAg, but also in HBsAg levels (Figure 2B). We defined group 1 as having low or undetectable HBeAg levels, and intermediate HBV DNA levels and HBsAg levels; group 2 as having low or undetectable HBeAg, and low HBV DNA and HBsAg levels; group 3 as having high levels of HBeAg, HBV DNA and HBsAg (Figure 2; Table 1). Patient groups did not differ with respect to age or gender.

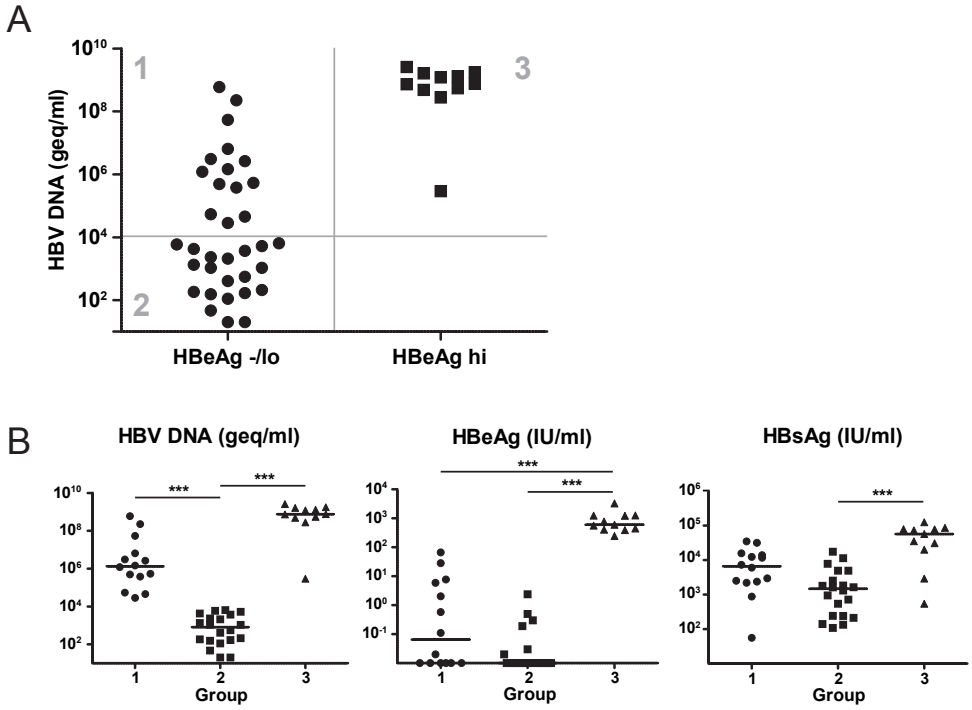

Figure 2. Chronic HBV patients divided into three groups based on HBV DNA and HBeAg levels. HBeAg levels, HBsAg levels and HBV DNA were measured in 45 chronic HBV patients. (**A**) HBV patients were divided into an HBeAgnegative/HBeAglow group (<100 IU/ml), and an HBeAghigh group (>100 IU/ml). Based on these two groups and HBV DNA levels, three groups of chronic HBV patients were defined (groups 1, 2 and 3). (**B**) Groups 1-3 were compared based on HBV DNA levels, HBeAg levels and HBsAg levels. *** p<0.0001, Kruskal-Wallis test, followed by Dunn's multiple comparison test.

Monocytes can be divided on the basis of their expression of CD14 and CD16, and these subpopulations have been reported to exert distinct functions [9,10]. To examine whether the chronic HBV patients in group 1, 2 and 3 differed in monocyte composition, we first determined the frequency of the monocytes expressing CD14 and CD16 in fresh peripheral blood samples from patients. As shown in Figure 3, the ratio of $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocytes did not differ between the distinct patient groups, indicating that chronic exposure to different amounts of HBV DNA, HBsAg and HBeAg did not lead to changes in the composition of the monocyte compartment.

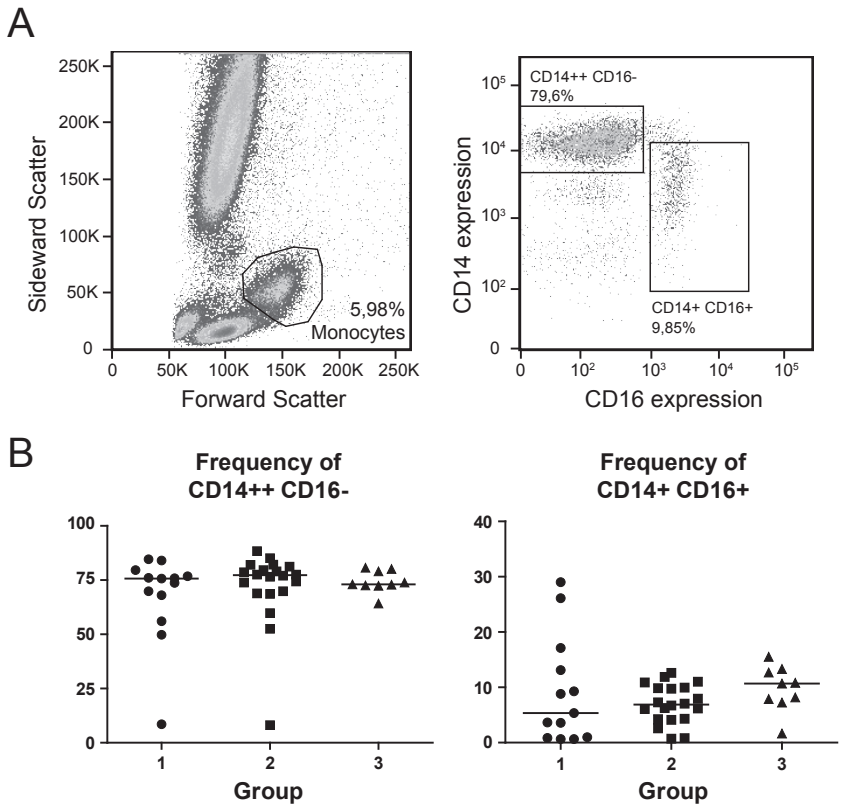

Figure 3. Different HBV DNA, HBeAg and HBsAg levels do not alter the frequency of monocyte subpopulations. Whole blood samples from chronic HBV patients were stained for CD14 and CD16 to analyze frequencies of the monocyte $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ subpopulations. (**A**) Gating strategy: monocytes were identified on the basis of their forward-sideward scatter, and divided into $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ populations. (**B**) The frequencies of monocyte subpopulations were compared between groups 1-3 (n=45).

Monocyte function does not differ between HBV patient groups

Since we showed that *in vitro* exposure of monocytes to HBsAg strongly induced the production of IL-6 and TNF, we explored whether the function of monocytes was affected by continuous exposure of monocytes to viral proteins in patients. To examine this, PBMC obtained from the patients with distinct serum HBV DNA, HBeAg and HBsAg levels were incubated overnight with medium or the TLR2 ligand Pam3CSK4, the TLR4 ligand LPS, or the TLR7/8 ligand R848, and cytokine production was measured by intracellular cytokine staining and ELISA. A high percentage of monocytes produced IL-6 and TNF after TLR stimulation (Figure 4A and 4B). As shown in figure 4B, TLR ligation of PBMC from chronic HBV patients with distinct serum levels of HBV DNA, HBeAg and HBsAg resulted in similar percentages of IL-6 and TNF-producing monocytes. This was observed for agonists against TLR2, TLR4 and TLR7/8. Importantly, also the baseline frequencies of cytokine-producing monocytes were not different between the three patient groups, suggesting that circulating monocytes from patients were not at a higher activation state as evidenced by spontaneous cytokine secretion. Next, detailed data-analysis using SPICE software was performed to evaluate the simultaneous production of multiple

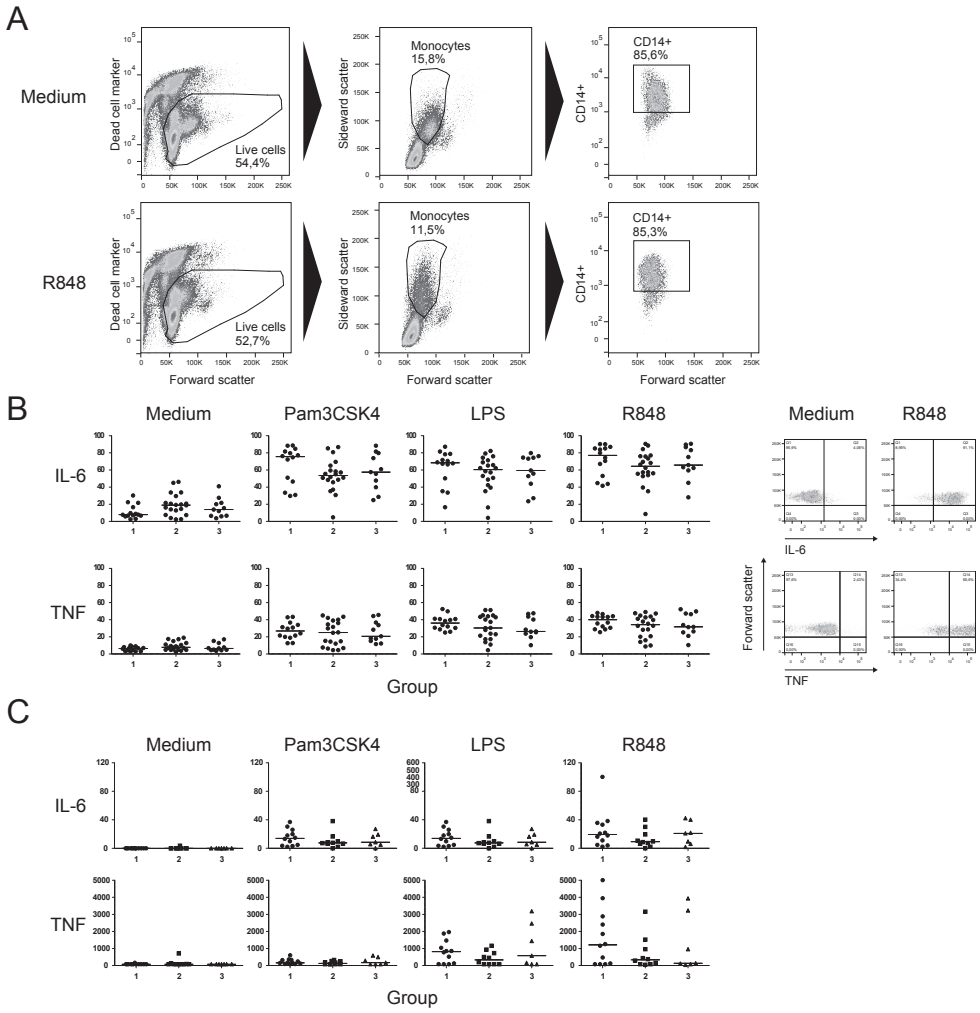

Figure 4. Monocytes from different patient groups display similar TLR-induced cytokine production. PBMC from patients with distinct HBV DNA, HBeAg and HBsAg profiles were incubated overnight with medium, Pam3CSK4, LPS or R848 to investigate the *in vivo* effect of HBV DNA levels, HBeAg levels and HBsAg levels on monocyte function. (**A**) Gating strategy: viable monocytes were identified on the basis of their forward-sideward scatter profile, and subsequently $CD14^+$ monocytes were gated to assess cytokine production by intracellular cytokine staining (n=45). (**B**, left) The frequencies of monocytes producing IL-6 and TNF upon medium or TLR ligand stimulation were compared between groups 1-3. (**B**, right) Representative dot-plots showing cytokine-positive cells induced by medium and R848 in one patient. (**C**) The cytokine levels measured in supernatant by ELISA (in ng/ml) upon stimulation of PBMC were compared between patient groups 1-3 (n=30).

cytokines by individual TLR-stimulated monocytes [17]. Again, no differences were observed when comparing the patient groups (data not shown). Besides frequencies, we also evaluated the intensity of the fluorescent signals representing the amounts of cytokines produced by monocytes upon stimulation, and also these parameters were not different between the patient groups (data not shown). In line with the above findings, also the amounts of TLR-induced IL-6 and TNF produced by monocytes, as measured by ELISA, were similar in the three groups of chronic HBV patients that differed in their serum levels of HBV DNA, HBsAg and HBeAg (Figure 4C).

Monocytes obtained from patients exposed *in vivo* to distinct levels of HBV DNA, HBeAg and HBsAg, react similarly to HBsAg stimulation *in vitro*

In contrast to the *in vitro* findings where exposure of monocytes to HBsAg leads to high cytokine induction, comparison of circulating monocytes obtained from patients with different levels of viral proteins *in vivo*, showed no higher spontaneous or TLR-induced cytokine induction. One possible explanation could be that continuous exposure of patient's leukocytes to HBV proteins makes them less sensitive to re-exposure. To examine this, we compared the *in vitro* effects of HBsAg on monocytes obtained from chronic HBV patients and from age- and sex-matched healthy individuals. Exposure of PBMC from patients to HBsAg resulted in IL-6 and TNF production, depicted by IL-6-positive (86.1± 2.0%) and TNF-positive (62.1± 6.6%) $CD14^+$ monocytes, while also monocytes from healthy individuals were positive for IL-6 (78.2± 5.4%) and TNF (55.4± 9.0%). Next, to examine whether monocytes from patients and controls were equally sensitive to the stimulatory effect of HBsAg, cells were stimulated with a dose-range of the HBsAg protein. As shown in Figure 5, monocytes from healthy controls and HBV patients were equally sensitive to HBsAg at low, intermediate and high doses of HBsAg, and consequently the responsiveness of monocytes to overnight HBsAg stimulation was similar between both groups. Likewise, HBsAg stimulation of PBMC for 5 hours, instead of 18 hours, also showed similar frequencies of IL-6 and TNF-producing monocytes between healthy controls and HBV patients, as shown in Figure S1. This supplementary figure shows not only stimulation with pHBsAg Ay as used throughout the paper, but also with recombinant HBsAg, both resulting in no difference between the groups. Also, assessment of overnight cytokine production by ELISA confirmed that monocytes from healthy individuals and from HBV patients react identical to HBsAg in terms of cytokine production (data not shown).

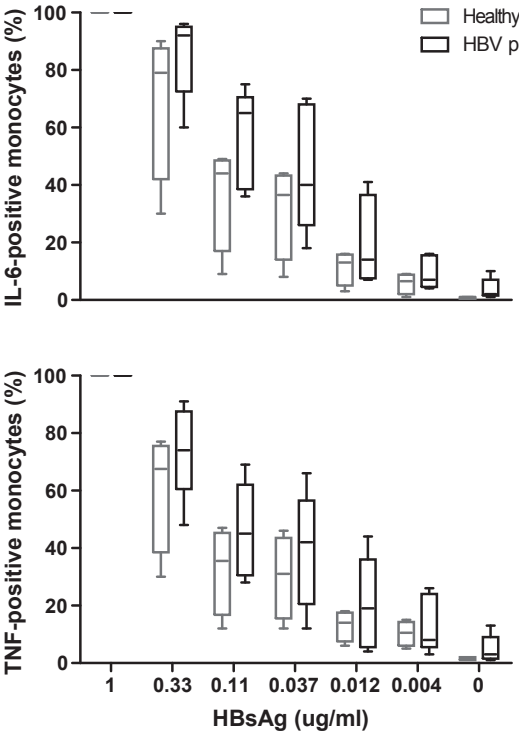

Figure 5. Monocytes from patients and healthy controls are equally sensitive to HBsAg *in vitro*. PBMC from healthy individuals or HBV patients with distinct *in vivo* exposure histories to HBV DNA, HBeAg and HBsAg (group 3) were stimulated with HBsAg and the relative frequency of cytokine-producing monocytes was determined (n=5-6). In the box-whisker plots, the line in the middle of the box is the median, while the whiskers depict the minimum and the maximum value.

IL-10 *in vitro* potently inhibits HBsAg-induced cytokine production by monocytes

Having demonstrated that monocytes from chronic HBV patients and healthy individuals are equally sensitive to HBsAg, we considered the activity of immunosuppressive cytokines, such as IL-10. In chronic HBV patients increased serum IL-10 levels have been demonstrated by various research groups [18-22]. Enhanced production of this immunosuppressive cytokine and increased sensitivity of activated monocytes to IL-10 may prevent monocyte activation by HBsAg *in vivo* [23,24]. As depicted in Figure 6, the presence of IL-10 clearly abrogated HBsAg-induced cytokine production, demonstrating a possible *in vivo* mechanism to curb HBsAg-induced cytokine production by monocytes.

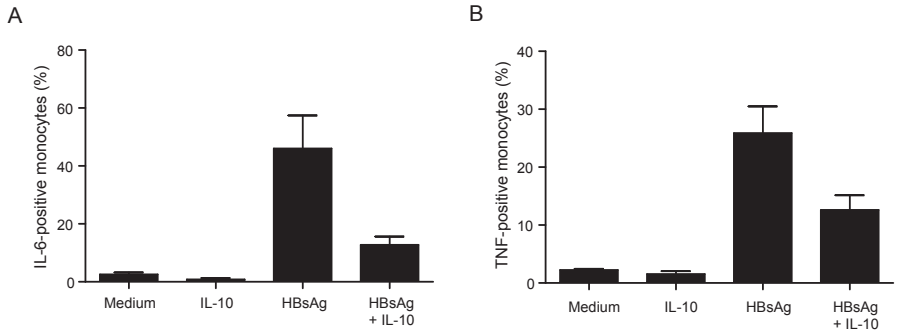

Figure 6. IL-10 inhibits the frequency of monocytes producing cytokines upon HBsAg exposure. PBMC from healthy individuals were stimulated with HBsAg, with or without IL-10. After overnight incubation, cells were stained for CD14 and intracellularly for cytokines. The frequencies of IL-6-positive (**A**) and TNF-positive (**B**) monocytes were compared between HBsAg and HBsAg and IL10 (n=3).

Discussion

In the present study, we demonstrate that HBsAg has a potent stimulatory effect on human monocytes upon *in vitro* culture. However, detailed comparison of monocytes obtained from chronic HBV patients showed that the differences in viral load, and levels of viral proteins did not influence the functionality of the monocyte compartment, suggesting that mechanisms are in place to prevent *in vivo* activation of monocytes by viral proteins such as HBsAg.

The majority of chronic HBV patients who are not being treated have relatively high levels of HBsAg in serum. We showed that monocytes are responsive to HBsAg, and get activated as a consequence of *in vitro* exposure to HBsAg as evidenced by the release of IL-6 and TNF. In agreement with our observations, others have demonstrated that patient-derived HBsAg induced the production of TNF and IL-10 by monocytes [16], and that monocyte-derived DC produced IL-12 upon stimulation with HBsAg [25]. However, there is controversy on the effect of HBsAg, since it was also reported that HBV, HBeAg and HBsAg are inhibitory and can in some cases suppress TLR-induced cytokine production in various cell types [26-30], including monocytes *in vitro* [31] and *in vivo* [13]. Importantly, using the same preparation of HBsAg, we previously demonstrated suppression of TLR9-induced IFNα by plasmacytoid DC as a consequence of exposure to HBsAg [29]. Furthermore, to rule

out that the stimulatory effects on monocytes were unique for the preparation, we tested different preparations from different suppliers and showed that the stimulatory capacity of HBsAg on monocytes was a general feature (rHBsAg (Prospec, Rehovot, Israel), pHBsAg Adr, pHBsAg Ayw (both Jena Bioscience GmbH, Jena, Germany), data not shown). This could mean that monocytes contribute to the initiation phase of an anti-viral immune response against HBV, being able to recognize the viral envelope protein and produce pro-inflammatory cytokines in the periphery, and possibly also in the liver during inflammation.

Since the HBsAg concentrations that were used for *in vitro* stimulations are in the same range as found in the serum of chronic HBV patients (5 µg/ml HBsAg corresponding to ±22,000 IU/ml HBsAg) [32], and since monocytes have been shown to interact *in vivo* with HBsAg [33], one might expect that this has a profound effect on the monocyte compartment in patients. Consequently, it is expected that patients with distinct levels of serum HBsAg, but also of HBV DNA and HBeAg, demonstrate an altered monocyte phenotype and function. Our findings show that the distribution of $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocytes was not different between patients with distinctive virological characteristics. Our findings appear not be in agreement with a study by Zhang *et al.*, which showed increased numbers of $CD16^{+}$ monocytes in immune-active HBeAg-positive patients as compared to healthy individuals or immunotolerant patients [12]. It should be noted that monocyte subset frequencies correlate with ALT values in chronic HBV patients [12], and that the study by Zhang et al, and ours differ considerably with respect to the ALT ranges included (13-1656 versus 15-443 IU/L). Therefore, differences in inclusion criteria of patients to address the respective research questions may likely explain the results. Additionally, we showed that also the functionality, as demonstrated by the frequency of TLR-induced cytokine producing monocytes and the TLR-induced cytokine production in PBMC was comparable in chronic HBV patients with different levels of viral DNA and viral proteins. This important finding on limited modulation of the monocyte compartment in chronic HBV patients *in vivo* is not reflected by the immunostimulatory effect of HBsAg on monocytes *in vitro*, and clearly demonstrates that comparison of the *in vivo* and *in vitro* findings is complex. It is also important to note that reported findings on the effects of HBsAg on immune cells use other cell types or cell lines than primary monocytes and different recombinant or plasma-derived preparations of HBsAg, which complicates the interpretation of the *in vitro* findings [26-31,34]. Our findings are in line with Gehring *et al.*, who showed that despite a constant exposure to HBsAg, ex vivo–isolated monocytes did not constitutively activate HBV-specific $CD8^{+}$ T cells [33] Evaluation of monocytes from chronic HBV patients showed that the induction of cytokines upon TLR ligation was not affected by the clinical phase. Furthermore, the spontaneous cytokine release of these monocytes was similar between all groups, suggesting that the continuous exposure to variable concentrations of HBV DNA, HBeAg or HBsAg did not result in modulation of the ex vivo activation state of these cells.

Combined these findings suggest that the monocyte compartment of patients is not affected as a consequence of exposure to different concentrations of viral proteins and HBV DNA in serum. Since this is in apparent conflict with the *in vitro* experiments in which monocytes were exposed to HBsAg, it is tempting to speculate that regulatory and/or compensatory mechanisms are in place *in vivo*, which prevent excessive activation of monocytes by serum HBsAg. We demonstrated that it is unlikely that unresponsiveness of patients' monocytes to HBsAg due to desensitization as a consequence of recent exposure to the same protein may explain the discrepancy

between the *in vitro* and *in vivo* observations, since monocytes from patients are similarly responsive to HBsAg as monocytes from healthy individuals, as shown by dose titrations. However, a likely candidate that may contribute to explain the *in vivo* data is the immunosuppressive cytokine IL-10 since it has been reported that chronic HBV patients have increased serum IL-10 levels [18-22]. Our data indeed show that IL-10 is able to inhibit cytokine production by HBsAg-induced monocytes, suggesting a possible role of IL-10 in restraining pro-inflammatory cytokine production by monocytes in HBV patients with high levels of HBsAg, HBeAg and HBV DNA. However, also other candidate molecules present in serum, such as TGFβ may further regulate monocyte function in chronic HBV patients.

In summary, we demonstrate that HBsAg has potent stimulatory effects on monocytes *in vitro*. However, in chronic HBV patients, the functionality of the monocyte compartment is not influenced by variations in the levels of serum viral components, suggesting that regulatory mechanisms are in place to prevent excessive *in vivo* activation of monocytes.

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Data supplement

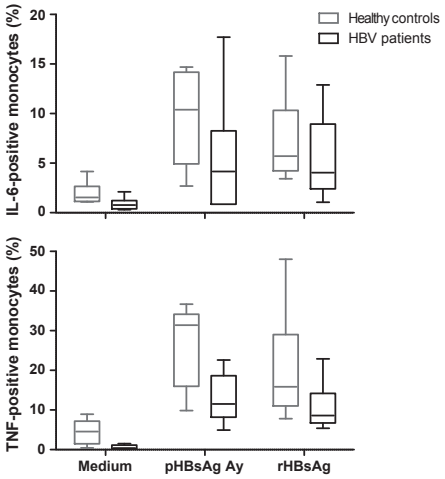

Figure S1. Monocytes from patients and healthy controls are equally sensitive to HBsAg *in vitro*. PBMC from healthy individuals or HBV patients with distinct *in vivo* exposure histories to HBV DNA, HBeAg and HBsAg (group 3) were stimulated for 5 hours with patient plasma-derived HBsAg or recombinant HBsAg and the frequency of cytokine-producing monocytes was determined (n=5-6). In the box-whisker plots, the line in the middle of the box is the median, while the whiskers depict the minimum and the maximum value.

Kupffer cells interact with HBsAg *in vivo* and *in vitro* leading to pro-inflammatory cytokine production and NK cell function

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Submitted

Chapter 6

Abstract

Background: Based on their localization, Kupffer cells (KC) likely interact with hepatitis B virus (HBV). However, the role of KC in inducing immunity towards HBV is poorly understood. Therefore, the interaction of hepatitis B surface antigen (HBsAg) and KC, and possible functional consequences, were assessed.

Methods: KC in liver tissue from chronic HBV patients were analysed for presence of HBsAg and for their phenotype, and compared to KC in control liver tissue. Liver graft perfusate-derived KC and *in vitro*-generated monocyte-derived macrophages were investigated for functional interaction with patient-derived HBsAg.

Results: Intrahepatic KC were positive for HBsAg and more activated than those from control livers. KC bound HBsAg *in vitro*, which did not change their phenotype, but strongly induced pro-inflammatory cytokine production. Additionally, monocyte-derived macrophages also bound HBsAg leading to activation and cytokine production. Furthermore, HBsAg-exposed macrophages and KC activated NK cells resulting in increased CD69 expression and IFNγ production.

Conclusions: KC directly interact with HBsAg *in vivo* and *in vitro*. HBsAg-induced function of KC and monocyte-derived macrophages and subsequent NK cell activation could be an early event in viral containment and the induction of HBV-specific immunity in the early phase after HBV infection, but could also contribute to liver pathology.

Introduction

Hepatitis B virus (HBV) can cause chronic liver disease and may elicit progressive liver injury leading to increased risk of developing liver cirrhosis, liver failure and liver cancer [1, 2]. The immunological mechanisms determining either the induction of effective anti-viral immunity leading to self-limiting hepatitis or the lack of effective immune response towards the virus leading to chronic hepatitis B are still unclear. Although recently an HBV receptor has been identified on hepatocytes [3], the early steps in the recognition of the virus by immune cells and the functional consequences of this interaction remain to be resolved.

Kupffer cells (KC) are the resident macrophages of the liver [4, 5] that besides being maintained by liver-derived precursors [6], have been shown to derive from monocytes [7] in an M-CSF dependent manner [5, 8]. They form, together with the sinusoidal endothelial cells, the first barrier for pathogens to enter the liver via the portal vein [9]. In humans, KC have been identified by the expression of CD68 and CD14 [10-12]. Their number and location, but also typical macrophage properties like endocytic capacity, expression of pattern recognition receptors (PRR), MHC and co-stimulatory molecules, and ability to produce cytokines upon stimulation [10, 13-18], potentially render KC effective immune cells contributing to either viral clearance or persistence.

Despite their potential, the exact role of KC in viral infection of the liver, and HBV infection in particular, is still unclear. On one hand KC are considered to contribute to intrahepatic tolerance as demonstrated by rat KC exposed to HBV that hardly expressed pro-inflammatory cytokines, but instead preferably produced TGFβ [19]. On the other hand, stimulation of human non-parenchymal cells with HBV was found to induce the production of the pro-inflammatory cytokines which was ascribed to KC-HBsAg interaction [20]. However, whether human KC are able to bind/take up HBV *in vivo* or *in vitro* is not known.

Therefore, we investigated the interaction between HBsAg and human KC by assessing the presence of HBsAg within the $CD14^+$ intrahepatic macrophage population and the phenotype of KC during HBV infection as well as the functional consequences of HBsAg-KC interaction *in vitro* using both liver-derived KC and *in vitro*-generated macrophages.

Materials and methods

Kupffer cell phenotype

Excess material of percutaneous needle liver biopsies obtained from 7 chronic HBV patients (Table 1) and 14 incisional biopsies obtained from donor liver during transplantation procedure was dissected into small pieces, incubated in RPMI culture medium (Lonza, Verviers Sprl, Belgium) containing 0.5 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO) and 0.1 mg/ml DNAse (Roche, Indianapolis, IN, USA), penicillin/streptomycin (Gibco, Carlsbad, CA, USA), L-glutamin (Lonza), and HEPES (Lonza) for 30 minutes, filtered through a 70 µm nylon cell strainer

(BD, San Diego, CA, USA) to acquire a liver cell suspension. Subsequently, cells were stained with antibodies against CD11b (ICRF44; BD Pharmingen, San Diego, CA, USA), CD16 (CB16; eBioscience), CD14 (61D3), CD40 (5C3), HLA-DR (LN3; all eBioscience), CD45 (2D1; BD), CD80 (MAB104; Beckman Coulter, Brea, CA, USA), CD86 (IT2.2), HLA-ABC (W6/32; both Biolegend, San Diego, CA, USA) in PBS supplemented with 1% human AB-serum (Lonza) and 0.02%NaN3. Data were acquired on a FACSCanto (BD) and analysed with FlowJo (Tree Star, Inc., Ashland, OR, USA).

Table 1

Patient characteristics

Patient #	Age (yr)	Sex	HBV genotype	Viral load (geq/ml)	HBeAg status	ALT (U/l)
1	49	F	ND	$4{,}78x10^{1}$	-	209
2	36	F	D	$7{,}01x10^{3}$	-	53
3	44	M	C	$6{,}63x10^{6}$	+	50
4	26	F	A	$3{,}55x10^{7}$	-	50
5	19	M	A	$1{,}68x10^{9}$	+	97
6	24	F	C	$1{,}05x10^{9}$	+	41
7	27	F	B	$2{,}86x10^{7}$	+	164

ND = Not determined; geq = genome equivalents

HBsAg staining on patient-derived blood and liver tissue

Excess material of percutaneous needle liver biopsies were obtained from 13 chronic HBV patients and 9 non-HBV liver disease patients (Table 2), collected in RPMI and filtered through a 70 µm nylon cell strainer (BD) to acquire a single-cell suspension. In parallel, peripheral blood was obtained from these patients and PBMC were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. Liver cells and PBMC were stained with antibodies against CD14 (MOP9) and CD45 (SK7; both BD) in PBS (Lonza)/1%FCS (Sigma)/0.02%NaN3, fixed with 2% formaldehyde, permeabilized with 0.5% saponin (VWR, West Chester, PA, USA) and stained using an antibody against HBsAg (recognizing subtypes HBsAg ad and ay; Acris Antibodies GmbH, Hiddenhausen, Germany) in 0.5% saponin (VWR). Data were acquired on a FACSCanto (BD) and analysed with FlowJo (Tree Star) and FACSDiva software (BD).

The medical ethical committee of the Erasmus MC University Medical Center declared to have no objections against the use of excess patient biopsy material and all patients with liver disease gave informed consent before inclusion.

Table 2

Patient characteristics

Patient #	Age (yr)	Sex	Disease	HBV genotype	Viral load (geq/ml)	HBeAg status	HBsAg status	ALT
HBV patients								
1	29	M	HBV	D	$4{,}17x10^{3}$	-	+	38
2	45	M	HBV	D	$5{,}12x10^{9}$	-	+	155
3	31	M	HBV	D	$6{,}46x10^{4}$	-	+	78
4	25	M	HBV	C	$8{,}48x10^{8}$	+	+	444
5	44	M	HBV	A	$3{,}05x10^{6}$	-	+	58
6	28	M	HBV	D	$2{,}50x10^{7}$	+	+	56
7	31	M	HBV	ND	$4{,}34x10^{3}$	-	+	28
8	39	F	HBV	D	$7{,}16x10^{5}$	-	+	30
9	26	F	HBV	E	$2{,}39x10^{3}$	-	+	41
10	40	M	HBV	ND	$2{,}25x10^{10}$	+	+	101
11	22	F	HBV	D	$2{,}14x10^{3}$	ND	+	86
12	32	F	HBV	D	$2{,}37x10^{8}$	+	+	46
13	27	F	HBV	ND	$2{,}27x10^{4}$	-	+	38
Non-HBV patients								
15	54	M	HCV	NA	ND	ND	-	160
16	64	F	HCV	NA	ND	ND	-	182
17	47	M	HCV	NA	ND	ND	-	16
18	43	M	Sjögren's syndrome	NA	ND	ND	-	63
19	52	F	PSC	NA	ND	-	-	281
20	41	M	Steatohepatitis	NA	ND	ND	-	43
21	45	M	HCV	NA	ND	-	-	59
22	22	M	HCV	NA	ND	ND	-	43
23	35	F	HCV	NA	ND	ND	-	78

HCV = Hepatitis C virus; PSC = Primary sclerosing cholangitis; ND = Not determined; NA = Not applicable; geq = genome equivalents; ALT = Alanine transaminase

Cell purification and culture

Liver mononuclear cells (LMNC) were isolated from liver graft perfusates as previously described [21]. LMNC were isolated from fresh perfusates derived from the second back table flush which contains minimal contamination with donor peripheral blood cells [21, 22]. LMNC were isolated using Ficoll-Paque (GE Healthcare) density gradient centrifugation, and subsequently frozen for future use. KC were isolated from LMNC using CD14 MACS microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) or using FACS sorting by selecting $CD14^{+}$ (M5E2; BD Pharmingen) cells using a FACSAria II (BD).

To generate monocyte-derived macrophages, PBMC were isolated from buffy coats from healthy blood donors (Sanquin) using Ficoll-Paque (GE Healthcare) density gradient centrifugation. Monocytes were isolated using CD14 MACS microbeads (Miltenyi Biotec). Macrophages were generated in 6-well plates (Costar, Corning, NY, USA) by culturing $1.5x10^6$ monocytes in 2 ml/well RPMI 1640 containing 10% heat-inactivated FCS (Sigma), penicillin/streptomycin (Gibco), L-glutamin (Lonza), and HEPES (Lonza), in the presence of 10 ng/ml M-CSF (R&D, Minneapolis, MN, USA). After 7 days monocyte-derived macrophages were harvested and used in experiments.

NK cells were isolated from LMNC or buffy coat-derived PBMC using an NK isolation kit (Miltenyi Biotec) or FACS sorting by selecting $CD3^-$ (UCHT1) $CD56^+$ (MY31; both BD) cells using a FACSAria II (BD).

In vitro binding/uptake of HBsAg

In vitro-generated macrophages ($5x10^4$/well) or LMNC ($1x10^6$/well) were cultured in 96-well plates in 125-150 µl RPMI 1640 containing 10% heat-inactivated FCS (Sigma), penicillin/streptomycin (Gibco), L-glutamin (Lonza), and HEPES (Lonza), at 37°C in the absence or presence of 2.5 µg/ml patient plasma-derived HBsAg serotype Ay (American Research Products (ARP, Waltham, MA, USA) that was unlabelled or directly labelled via conjugation of maleimide-activated DyLight 650 (Thermo Fisher Scientific, Rockford, IL USA) to free reduced sulfhydryl (-SH) groups, according to manufacturer's protocol. After conjugation unbound conjugate was removed by dialysis against PBS as dialysis buffer, using dialysis units of pore size 20,000 kDa (Thermo Fisher Scientific). After 1-4 hours, cells were harvested, fixed with 2% formaldehyde, permeabilized with 0.5% saponin (VWR), and either stained with biotin-labeled goat polyclonal anti-HBsAg Ad/Ay (Abcam, Cambridge, UK), and Streptavidin-PerCP (BD) or directly stained for surface marker CD14 (61D3) and CD3 (SK7; both eBioscience), and subsequently analysed by flow cytometry as described above.

HBsAg stimulation of Kupffer cells and *in vitro*-generated macrophages

Liver perfusate-derived KC or *in vitro*-generated macrophages were cultured in 96-well plates in 250 µl RPMI 1640 containing 10% heat-inactivated FCS (Sigma), penicillin/streptomycin (Gibco), L-glutamin (Lonza), and HEPES (Lonza), in the absence or presence of 2.5 µg/ml plasma-derived HBsAg serotype Ay (pHBsAg; ARP) or CHO-derived recombinant HBsAg (rHBsAg; Prospec, Rehovot, Israel) for 48 hours. Supernatants were harvested, and cytokine production (IL-6, TNF and IL-10) was determined by ELISA (all kits from eBioscience). After removal of supernatants, cells were harvested, stained for CD40 (5C3; eBioscience), CD80 (MAB104; Beckman Coulter), and CD86 (IT2.2; Biolegend) and analysed by flow cytometry as described above.

HBsAg stimulation of LMNC: intracellular cytokine staining

LMNC ($1x10^6$ cells/ml) were cultured in 96-well plates (Corning, Lowell, MA, USA) in 250 µl X-VIVO culture medium (Lonza, Verviers Sprl, Belgium) containing penicillin/streptavidin (Gibco), L-glutamin (Lonza), and HEPES (Lonza) for 18 hours at 37°C, 5% CO_2. Cells were either unstimulated or stimulated with 2.5 µg/ml human plasma-derived HBsAg (ARP). During the last 16 hours of culture, brefeldin A (10 µg/ml; Sigma-Aldrich) was added. Cells were harvested, incubated with LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen, Ltd., Paisley, United Kingdom), fixed with 2% formaldehyde, permeabilized with 0.5% saponin (VWR) and stained for IL-6 (MQ2-13A5), TNF (Mab11; all eBioscience), CXCL8 | IL-8 (6217), IL-15 (34559; both R&D), IL-10 (JES3-19F1 ; BioLegend), IL-12p40 (C11.5) or CCL4 | MIP-1β (D21-1351; both BD Pharmingen) and CD14 (61D3) and CD45 (HI30; both eBioscience), and analysed by flow cytometry as described above.

Macrophage-NK cell co-culture

$1x10^5$ liver perfusate-derived KC or *in vitro*-generated macrophages were co-cultured with autologous perfusate-derived NK cells or peripheral blood NK cells, respectively, in a 1:1 ratio in 48-well plates in 250 µl RPMI 1640 containing 10% heat-inactivated FCS (Lonza), penicillin/streptomycin (Gibco), L-glutamin (Lonza), and HEPES (Lonza) either with or without 2.5 µg/ml pHBsAg-Ay (ARP). After 48 hours, supernatants and cells were harvested. Cells were stained with antibodies against CD3 (UCHT1), CD56 (MY31), and CD69 (L78; all BD) and analysed by flow cytometry as described above. Supernatants were assessed for IFNγ production by ELISA (eBioscience, detection limit 4 pg/ml) according to manufacturer's protocol.

Results

Kupffer cells display an activated phenotype in chronic HBV and interact with HBsAg *in vivo*

To investigate the effect of chronic hepatitis B virus infection on KC, we compared the phenotype of $CD45^+CD14^+$ cells present in liver biopsy tissue of chronic HBV patients with control liver tissue derived from healthy transplant donor livers. The percentages of KC expressing CD11b, CD11c, CD163 and CD204 were comparable between both groups (Fig. 1A), albeit that the expression levels of CD11c and CD163 were significantly higher on KC from HBV patients than on those of HC (Supplementary Fig. 1B, $p<0.05$). Only the percentage of CD16-expressing KC was significantly lower in chronic HBV compared to healthy control livers (Fig. 1A). With regard to the expression of activation markers, we observed that KC present in control liver tissues showed a low expression of CD40, which differed between donors, and were almost negative for CD80, whereas they expressed high levels of CD86, MHC class I and II molecules (Fig. 1B). The activation status of KC in HBV-infected livers was found to be increased as demonstrated by significantly increased expression of CD40, HLA-ABC and HLA-DR and also the expression of

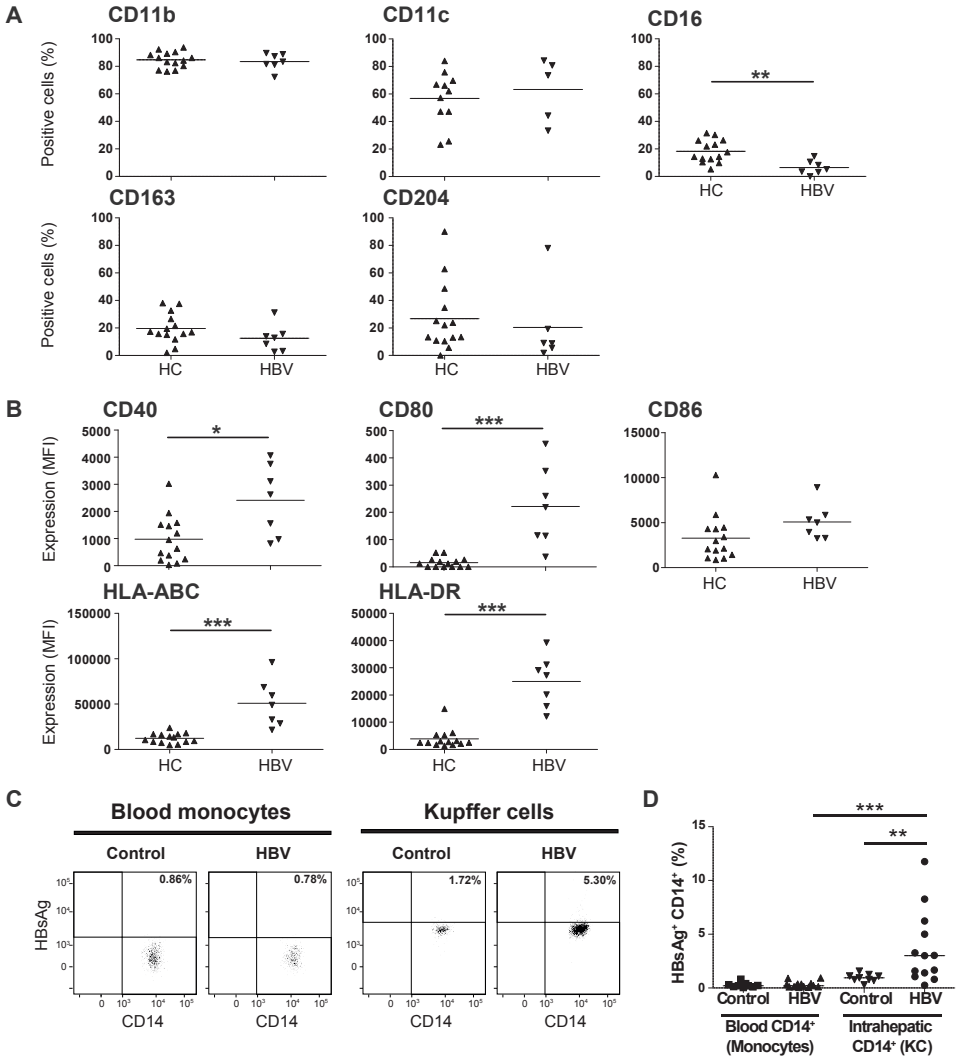

Figure 1. Kupffer cells in chronically HBV-infected livers. KC phenotype (**A**) and activation status (**B**) was determined in liver biopsy samples from HC (n=11-14) and HBV patients (n=5-7) by flow cytometry. Expression is shown as percentage positivity on $CD14^+$ cells (**A**) or as MFI with background staining subtracted (**B**). $CD14^+$ peripheral blood monocytes and KC were analysed for HBsAg-positivity in blood and liver biopsy samples of CHB patients (n=13) and non-HBV-related liver disease patients (n=9) by flow cytometry. Shown are representative dot plots (**C**) and individual and mean HBsAg-positivity of all patients (**D**). *p<0.05, **p<0.01, ***p<0.001, Mann-Whitney test.

CD86 tended to be increased (Fig. 1B).

We next analysed blood and liver biopsy samples from either chronic HBV patients or patients with non-HBV related liver disease for presence of HBsAg. Whereas a small, but significant, percentage of KC from chronic HBV patients stained positive for HBsAg, peripheral blood $CD14^+$ monocytes from the same patients did not. As expected, $CD14^+$ cells derived from non-HBV patients were negative for HBsAg (Fig. 1CD). These data clearly indicate that KC have the ability to interact with HBsAg *in*

vivo and that $CD45^+CD14^+$ cells present in HBV-infected livers are significantly more activated than those present in healthy control livers.

HBsAg interacts with KC *in vitro* inducing cytokine production

To further investigate this KC-HBsAg interaction, LMNC or isolated KC were incubated with pHBsAg *in vitro*. After incubation of LMNC, very high HBsAg-positivity was seen in $CD14^+$ KC (90%±6.4%), while $CD3^+CD56^-$ T cells, used as a control population within the same LMNC population, showed hardly any HBsAg-binding (Fig. 2AB). These data show the high potential of KC to interact with pHBsAg. To investigate whether the activated phenotype of KC in chronic HBV patients could be ascribed to a direct effect of HBsAg, liver graft perfusate-derived KC were isolated and cultured either in presence or absence of pHBsAg for 48 hours. These KC expressed high levels of CD11b, intermediate to high levels of CD163 varying widely between donors (range 18.5 – 98.7% $CD163^+$ KC), and low levels of CD204 (Supplementary Fig. 2A). Expression of activation markers on perfusate-derived KC was very similar to KC present in healthy control liver tissue (Supplementary Fig. 2B, data not shown). Neither exposure to pHBsAg nor exposure to rHBsAg changed the expression of co-stimulatory molecules (Fig. 2C and data not shown), MHC molecules, or differentiation markers CD11b, CD163 and CD204 (data not shown).

To assess the effect of HBsAg on cytokine and chemokine production, LMNC were cultured in the absence or presence of pHBsAg and $CD14^+$ KC were measured for intracellular cytokine expression. HBsAg induced many KC to produce the pro-inflammatory cytokines IL-6, TNF and chemokines CCL4 and CXCL8 in all donors tested, while it weakly stimulated IL-10 and IL-15 expression (Fig. 2D). Also the percentage of IL-12p40-producing KC was weakly enhanced in 4/5 experiments with different donors (Fig. 2D). To confirm the direct effect of HBsAg on KC, KC were isolated from these perfusate-derived LMNC and assessed for HBsAg-induced cytokine secretion. Both pHBsAg and rHBsAg stimulated KC to secrete TNF, IL-10 and copious amounts of IL-6 (Fig. 2E and Supplementary Fig. 2C). Together, these data show that HBsAg is able to interact with KC and induce pro-inflammatory cytokine production without inducing an activated phenotype based on co-stimulatory molecule and MHC expression.

HBsAg also functionally interacts with *in vitro*-generated macrophages

KC are thought to originate from liver precursor cells as well as from monocytes in an M-CSF dependent manner [5, 7, 8]. Since monocyte-derived macrophage development likely increasingly occurs during inflammation, a situation often present in HBV-infected livers, we also investigated the effect on M-CSF-driven human monocyte-derived macrophages. Compared to perfusate-derived KC, *in vitro*-generated macrophages expressed similar levels of CD11b, CD40, CD80 and HLA-DR, higher levels of CD163, CD204 and CD86 and lower HLA-ABC (Supplementary Fig. 3AB). Upon pHBsAg exposure *in vitro*-generated macrophages interacted actively with HBsAg as demonstrated by a high percentage of HBsAg-positive cells (Fig. 3A). In contrast to KC, incubation of *in vitro*¬-generated macrophages with pHBsAg induced an activated phenotype showing increased CD40 and CD80

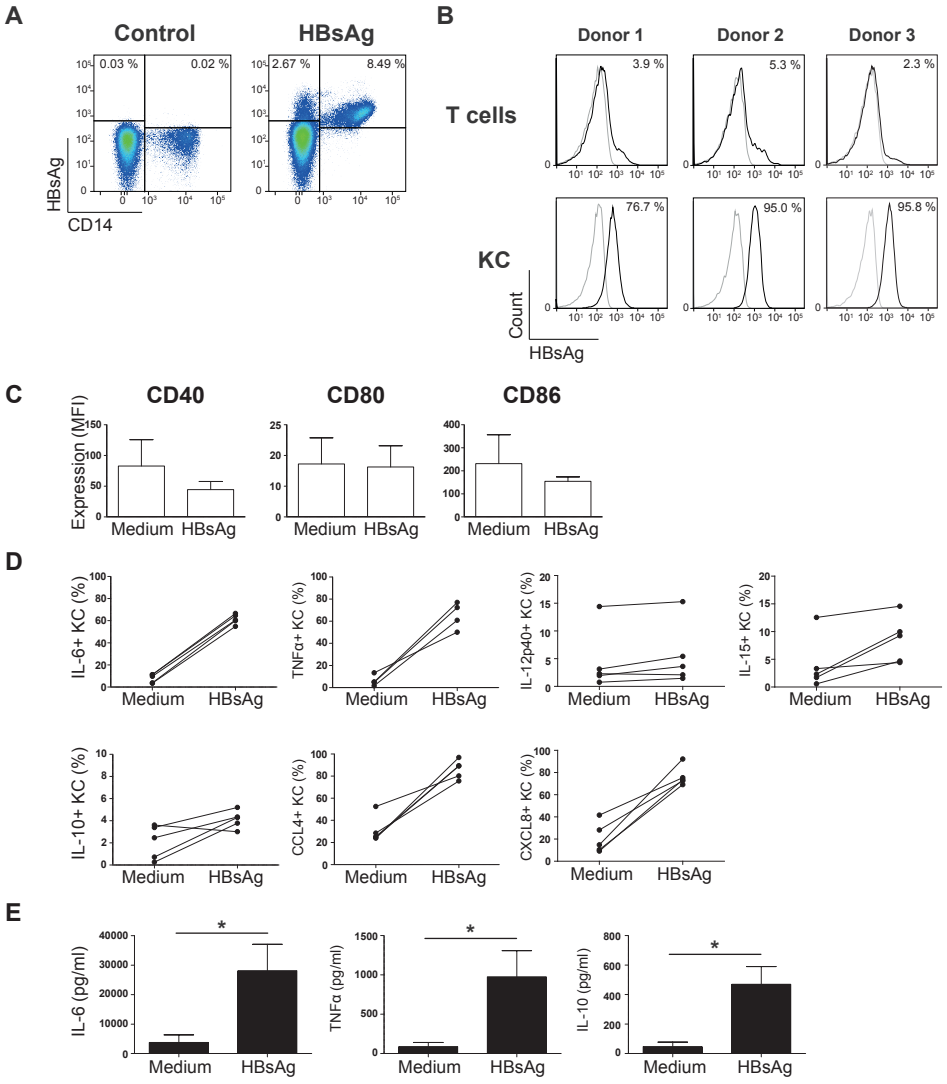

Figure 2. HBsAg interacts with KC *in vitro* inducing cytokine production. LMNC were incubated with/without fluorescent pHBsAg (1h) and analysed for HBsAg-positivity in $CD14^-$ and $CD14^+$ cells by flow cytometry. Shown are representative pseudo-colour density plots (**A**) and HBsAg-positivity in KC (CD14) and T cells (CD3) derived from different donors (**B**). (**C**) Isolated KC were cultured with/without pHBsAg for 48 hours. Their activation status was assessed by flow cytometry and expressed as mean±SEM MFI (n=4-5). (**D**) Intracellular cytokine-positive KC within LMNC exposed to pHBsAg or medium per donor as assessed by flow cytometry (n=5) (**E**) Cytokine production by isolated KC cultured with or without pHBsAg for 48h as determined by ELISA on supernatants (mean±SEM, n=8). *p<0.05, **p<0.01; Wilcoxon signed rank test.

expression, and decreased CD86 expression (Fig. 3B), besides inducing IL-6, TNF and IL-10 production (Fig. 3C).

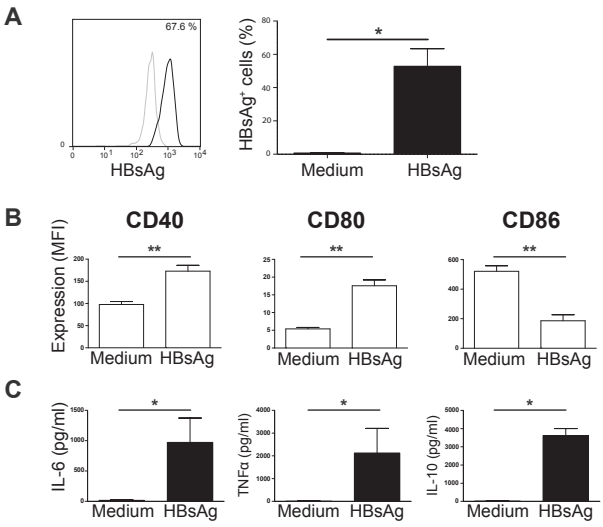

Figure 3. Interaction of monocyte -derived macrophages with HBsAg. Monocyte-derived macrophages were incubated with/ without pHBsAg. (**A**) After 4 hours HBsAg positivity was assessed by flow cytometry, as shown in a representative histogram overlay (left panel) and as mean±SEM of 6 independent experiments with different donors (right panel). The effect of 48h culture in the absence or presence of pHBsAg on *in vitro*-generated macrophage on the (**B**) expression of co-stimulatory molecules (n=9) and (**C**) cytokine secretion (n=9) as assessed by flow cytometry and ELISA, respectively. Data are shown as mean±SEM. *p<0.05; **p<0.01, Wilcoxon signed rank test.

HBsAg-activated macrophages induce NK cell function

To further examine the functional consequence of the interaction between HBsAg and macrophages, we investigated the interaction of KC and *in vitro*-generated macrophages with autologous liver-derived NK cells and peripheral blood-derived NK cells, respectively. When culturing *in vitro*-generated macrophages together with NK cells in the presence of pHBsAg, NK cells not only strongly upregulated CD69 expression (Fig. 4A), but also significantly increased IFNγ production (Fig. 4B). The basal CD69 expression of liver NK cells is already high, as demonstrated before [23] and could hardly be further increased upon exposure to HBsAg-stimulated KC (Fig. 4C). Also for the KC-NK cell co-cultures, induction of IFNγ production by NK cells was demonstrated (Fig. 4D). Adding pHBsAg to NK cells, KC or macrophages alone neither changed CD69 expression nor induced IFNγ production (data not shown).

Discussion

A virus-induced innate immune response is generally considered to be crucial for early viral containment and the induction of virus-specific immunity. However, due to delayed onset of symptoms in HBV-infected humans and lack of appropriate animal models, HBV-induced innate immune responses are largely undefined. We demonstrated, for the first time, a direct interaction between HBsAg and KC both *in vivo* and *in vitro*. The interaction of KC as well as *in vitro*-generated monocyte-derived macrophages with HBsAg resulted in cytokine production and the induction of IFNγ production by NK cells.

The finding of HBsAg-positive KC in HBV-infected livers together with the production of cytokines, and the induction of NK cell function, supports the hypothesis that

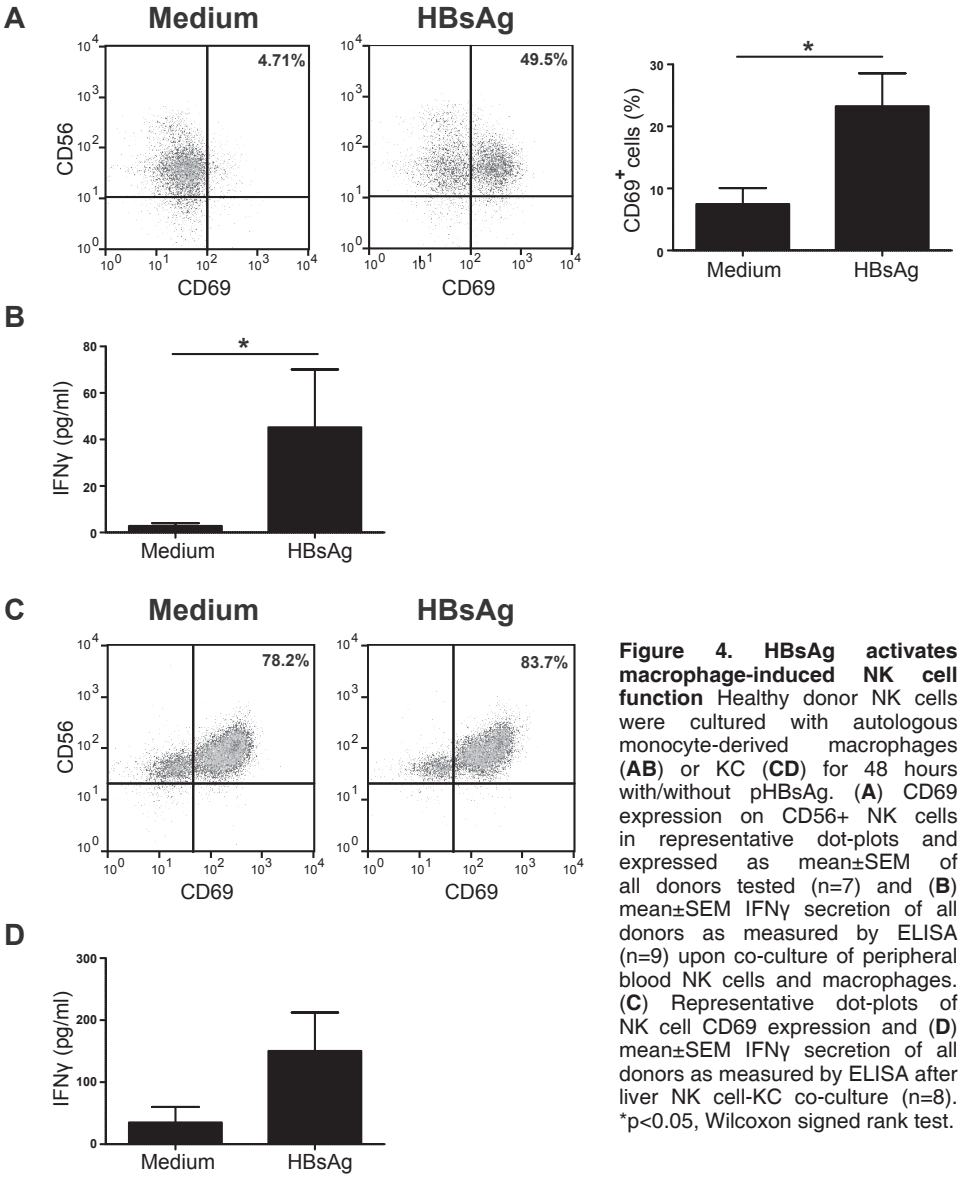

Figure 4. HBsAg activates macrophage-induced NK cell function Healthy donor NK cells were cultured with autologous monocyte-derived macrophages (**AB**) or KC (**CD**) for 48 hours with/without pHBsAg. (**A**) CD69 expression on CD56+ NK cells in representative dot-plots and expressed as mean±SEM of all donors tested (n=7) and (**B**) mean±SEM IFNγ secretion of all donors as measured by ELISA (n=9) upon co-culture of peripheral blood NK cells and macrophages. (**C**) Representative dot-plots of NK cell CD69 expression and (**D**) mean±SEM IFNγ secretion of all donors as measured by ELISA after liver NK cell-KC co-culture (n=8). *p<0.05, Wilcoxon signed rank test.

KC play a role in HBV recognition and immune activation in the early phase after infection. Whether the HBsAg acquired by intrahepatic KC is the result of a direct interaction between HBsAg as part of the whole HBV virion, HBsAg as circulating subviral particle, or the result of the uptake of HBV/HBsAg-containing hepatocytes remains to be elucidated. Although serum HBsAg levels were not determined, we did not find a correlation between serum HBV-DNA levels and HBsAg-positivity in KC (data not shown).

The relative low percentage and staining intensity of the HBsAg$^+$ KC in HBV-infected livers compared to the signals obtained from KC-HBsAg interaction *in vitro* seems

counter-intuitive, but could have several explanations. First, HBsAg$^+$ KC detection by using anti-HBsAg antibody instead of directly fluorescent HBsAg used *in vitro* was significantly lower (data not shown). Additionally, it is tempting to speculate that the longer exposure time of KC to HBsAg *in vivo* may not further increase the level of antigen uptake, but rather impairs HBsAg detection since it is known that HBsAg, once taken up, will be broken down by macrophages [24]. Furthermore, the higher activation status of KC present in these chronically infected livers may negatively influence the uptake capacity of macrophages as has been shown for dendritic cells, cells closely related to macrophages [25, 26].

The receptors involved in KC-HBsAg interaction will be subject of future research, but several candidate receptors exist. CD14 has been demonstrated as an interesting candidate receptor on monocytes. Recently, Gehring *et al.* demonstrated HBsAg-positivity in CD14$^+$ monocytes of chronic HBV patients, using a very sensitive Tissuefax system due to the low quantity of HBsAg in these cells [27]. Due to a possible less sensitive detection method as discussed above, we cannot rule out the presence of HBsAg$^+$ monocytes and therefore also the role of CD14 in HBsAg binding cannot be excluded. Another possible receptor involved in HBsAg-KC interaction not expressed by monocytes is the macrophage mannose receptor, known to be involved in HBsAg interaction with dendritic cells [34]. Altogether, it seems plausible to conclude that KC contain higher levels of HBsAg than circulating monocytes, which could be explained by cell-intrinsic properties of macrophages and/or higher levels of HBV/HBsAg in the intrahepatic compartment.

The absence of HBsAg-induced upregulation of co-stimulatory and MHC molecules on KC *in vitro* suggests that indirect activation is responsible for the activated phenotype of KC in HBV-infected livers. However, since HBsAg exposure of *in vitro*-generated monocyte-derived macrophages induced an activated phenotype, an alternative explanation could be that the activated phenotype observed is the result of activated infiltrating monocyte-derived macrophages since an increased differentiation of monocytes into macrophages is likely to occur during inflammatory responses [28-30]. As monocytes, monocyte-derived macrophages and KC share many surface markers it is hard to discern between these cell populations.

Although HBsAg did not directly affect KC phenotype *in vitro*, HBsAg induced production of high levels of pro-inflammatory cytokines by these cells. The HBsAg-induced production of IL-6, TNF and CXCL8 by KC is comparable with the HBV-induced production of these cytokines by non-parenchymal liver cells, presumably KC, reported by Hösel *et al.* These cytokines are possibly beneficial for the early viral containment, since cytokines like IL-6 and TNF directly inhibit HBV replication [20, 31], and could provide the first signals towards the initiation of HBV-specific immunity in the early phase after HBV infection by attracting and activating other immune cells. The IFNγ production by NK cells upon co-culture with HBsAg-activated macrophages is likely mediated by KC-derived cytokines such as IL-12 and IL-15 [10]. Interestingly, also in WHV, intrahepatic IFNγ and IL-12 expression were found in the early phase after infection, and also the chimpanzee model of HBV demonstrated intrahepatic IFNγ levels prior to clear onset of the adaptive T cell response [32, 33]. Although we cannot completely rule out a contribution of T cell-derived IFNγ in these animal models, elevated NK cell frequencies have been reported in acute HBV patients [34]. Together with the cytokines produced by the KC themselves, these NK cell-derived factors may further control viral replication and will support the induction of virus-specific immunity [35]. And although KC were recently described to restrict liver

damage [36], these cytokines could also be harmful contributing to liver inflammation and liver fibrosis [37, 38].

In conclusion, the present study shows a direct interaction between KC and HBsAg, *in vivo* and *in vitro*. HBsAg-induced KC function and subsequent NK cell activation, could be an early event in the inhibition of HBV replication and induction of HBV-specific immunity during the early phase after HBV infection, but may also contribute to liver damage during the chronic phase.

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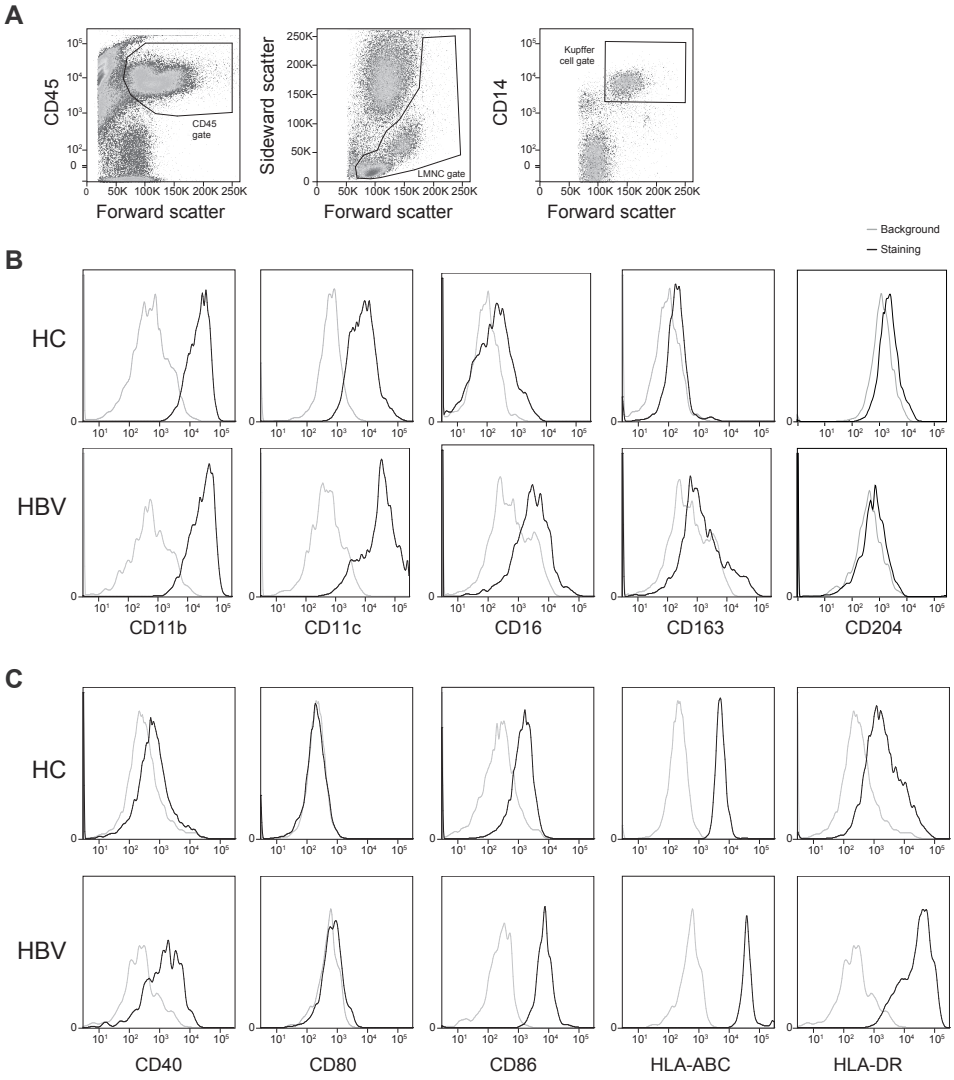
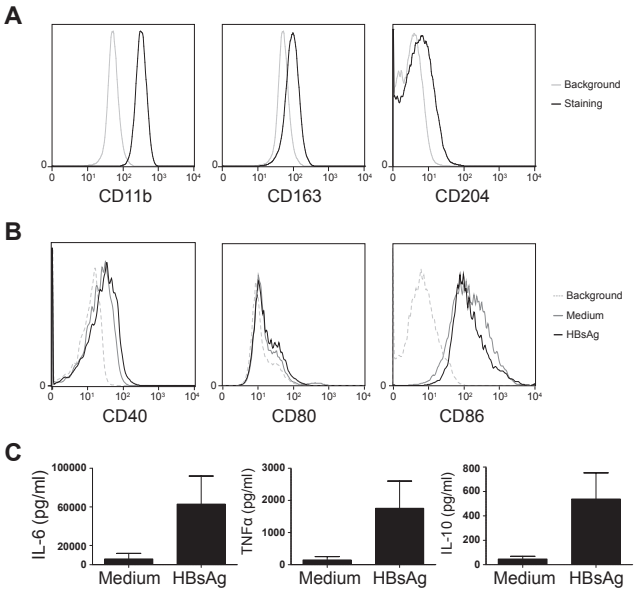
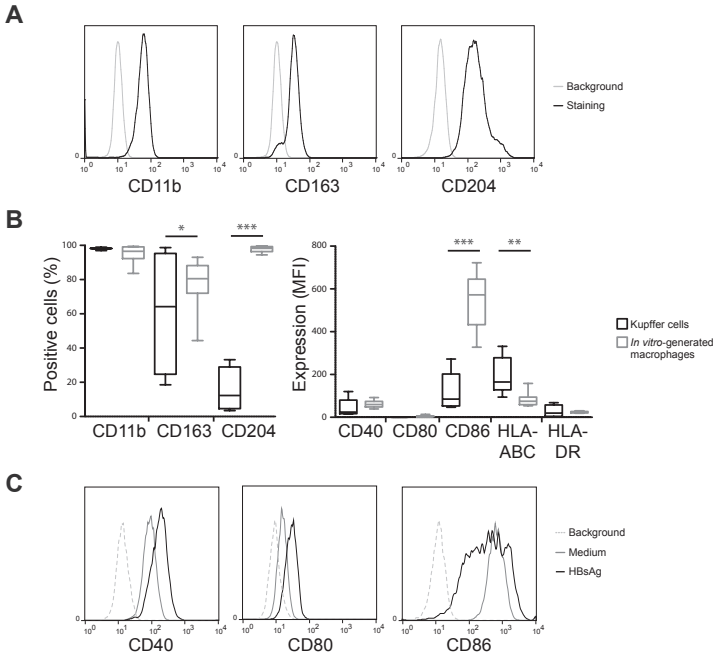
Data supplement



Supplementary Figure 1. Kupffer cells display an activated phenotype in chronic HBV. The phenotype of KC present in healthy control liver tissue (HC; n=14) and chronic HBV patients (n=7). (**A**) Representative pseudo-colour density plots of liver biopsy cells stained for CD45 and CD14 demonstrates the KC gating strategy used to perform flow cytometric analysis, which is based on CD45-positivity, FFS/SSC-gating to identify liver mononuclear cells and high CD14 expression. (**BC**) Representative histograms demonstrating the surface expression of different molecules on KC as assessed by flow cytometry as described in A. Shown are overlays of specific (black) and background staining (grey).



Supplementary Figure 2. Perfusate-derived KC phenotype and expression of co-stimulatory molecules and cytokines after HBsAg exposure. (**A**) Perfusate-derived KC were isolated and assessed by flow cytometry to determine their phenotype. Representative histograms are shown (n=6). (**B**) Isolated perfusate-derived KC were cultured with pHBsAg. After 48h, KC were assessed for their activation status by flow cytometry of which representative overlays are shown (n=6) (**C**) Isolated perfusate-derived KC were cultured with rHBsAg. After 48h, KC were assessed for cytokine production (mean±SEM) as assessed by ELISA (n=3).



Supplementrary figure 3. *In vitro*-generated macrophage phenotype, and expression of co-stimulatory molecule expression after HBsAg exposure. *In vitro*-generated macrophages were assessed by flow cytometry to determine their phenotype. (**A**) Representative histograms demonstrating the phenotype of monocyte-derived macrophages (n=10). (**B**) Comparison of isolated liver graft perfusate-derived KC (n=5) and *in vitro*-generated macrophages (n=10) phenotypes. Shown are box and whiskers plots, whiskers representing smallest and largest value, line in the middle representing median (**C**) Monocyte-derived macrophages were cultured with/without pHBsAg and assessed for their activation status by flow cytometry. Histograms demonstrated are representative for 9 independent experiments with different donors.

General discussion and summary

Chapter 7

Introduction

Chronic HBV infection research was and still is focused mainly on the adaptive immune system, albeit the last ten years there has been a steady but slow increase in papers on antigen-presenting cells. As discussed by Bertoletti *et al.*, coordinate activation of innate and adaptive response is necessary for HBV control [1]. Without a proper immune reaction by innate immune cells like DC, monocytes and macrophages, the ensuing adaptive immune response will be severely hampered [2, 3]. In this thesis we explored two aspects of the role of APC in HBV infection: firstly, we looked into effects of IFN on the function and phenotype of APC (chapter 3 and 4); secondly, we looked at the interaction of APC and viral proteins, most importantly HBsAg (chapters 5 and 6). Although, per chapter experiments and findings have been reviewed in each chapter's own discussion, some remaining topics will be discussed below. Ultimately, the consequences of some of the findings in this thesis and the future perspectives for this part of the field will be addressed.

Part I: Effect of *in vivo* and *in vitro* IFN on antigen-presenting cells

The mode of action of ribavirin

IFN until recently have been the mainstay of chronic hepatitis B treatment and hepatitis C treatment. Likewise, ribavirin has been a critical component of the standard of care combination therapy of IFN and ribavirin in the treatment of chronic hepatitis C infection for almost 20 years. Despite the extensive use of ribavirin in the treatment of HCV, the mechanism by which it executes its antiviral function remains unknown. Several mechanisms have been proposed, some of which we discussed in chapter 3. However, none convincingly accounts for the boost that it gives to IFN-based therapy as reviewed in [4]. Indeed, one would expect mechanisms like (1) the inhibition of inosine 5'-monophosphate dehydrogenase (IMPDH), an enzyme important in the de novo synthesis of guanosine [4], to affect a broad spectrum of especially RNA viruses. However, since GTP is a necessary component of viral RNA synthesis and HBV replicates via an RNA intermediate, ribavirin would be expected to inhibit HBV replication also. The lack of clinical efficacy of ribavirin monotherapy in HCV [5] and of the addition of ribavirin to IFN monotherapy in HBV [6] suggests that mechanisms like (1) inhibition of IMPDH, (2) the induction of lethal viral mutagenesis, (3) impairment of translation via eIF4E, and (4) direct inhibition of viral RNA-dependent RNA-polymerases, are not important mechanisms employed by ribavirin in this case [4]. Even though the latter mechanism is HCV-specific and ribavirin might have different direct effects on HBV. Because ribavirin showed a lack of clinical effect in chronic HBV patients, we were able to research immunological effects of adding ribavirin independently of a simultaneous decrease in HBV-DNA, assessing some of the other proposed modes of action of ribavirin. Another (5) mechanism proposed for ribavirin is the modulation of the T_H1/T_H2 lymphocyte balance [4], which does not take place in the treatment group containing ribavirin in our study, as addressed in chapter 3. Additionally, IFN-based therapy led to a strong decline of IFNγ-producing T_H1 cells. In accordance with these data, it has been suggested that it is unlikely that restoration of T cell responses is causal to an early response or SVR to therapy [7]. A different possible immune-modulatory effect

of ribavirin is (6) the modulation of the ISG expression. One of the ways that ribavirin, as a purine analogue, can exert such modulation was already discussed in chapter 3: direct activation of TLR7 and/or TLR9, or potentiation of the response to TLR7 and/or TLR9 did not occur in our study. However, another way of modulating the ISG expression is by improving the IFN signalling cascade. Especially when ribavirin was used in conjunction with IFNα, induction of specific ISG was synergistic as compared to IFN or ribavirin monotherapy [8]. How exactly this takes place, remains to be resolved. An interesting recent addition to this mechanism suggests that ribavirin seems to potentiate the susceptibility for IFN signalling by lowering the baseline expression of some ISG [9]. Since in our study we did not focus on this mechanism of ribavirin action, it is hard to draw conclusions or even speculate based on the data presented in chapter 2. The ribavirin-induced increase of co-stimulatory molecules suggests that ribavirin enhances IFN effects since IFN is known to increase the expression of CD40, CD80 and CD86 [10, 11]. However, if ribavirin potentiates IFN action by augmenting ISG induction, one might not expect that this effect also improves clinical efficacy in chronic HBV infection. A possible explanation for this could be that ribavirin amplifies the expression of specific ISG important in anti-HCV immunity, but unimportant in anti-HBV immunity. Another explanation could be that IFN signalling in HBV is more impaired than that in HCV, resulting in differential IFN effects, with or without ribavirin. Since the IFN-induced effects on co-stimulatory molecules, and perhaps also other IFN-induced factors, are limited in our study, it is not surprising that a ribavirin-induced enhancement is minimal.

Importantly, the type of cell in which certain ISG are upregulated seem to be pivotal for treatment response. In the case of ISG15, non-responders mainly show an upregulation of this ISG in hepatocytes, while upregulation of the same ISG is found primarily in KC of responders [12]. This shows us two important things: (A) modulation of the innate immunity by ribavirin may well be as important, if not more important, in resolving HCV infections than its effect on the intracellular immune response; and (B) the importance of cell type-specific induction of ISG, which will be addressed further in the type III IFN paragraph. Concerning the modulation of the innate immunity: in our study we showed an increase in the basal activation status of peripheral DC upon ribavirin addition. An increase in the activation status of DC may mean that these DC are functionally altered. Although not apparent in our study, changes in activation markers on pDC have been correlated to clinical outcome in HCV treatment [13]. Furthermore, the induction of DC-mediated T-cell proliferation and IFNγ production, and pDC-derived IFNα production was not increased in our study, there are other functionalities like the induction of T-cell or NK cell cytotoxicity that we have not looked at. Perhaps more importantly, in our study we looked at the effects of ribavirin on peripheral leukocytes, and we can therefore not exclude that the effects on DC and other immune cells like KC and NK cells migrating to or found in the liver may be more potent. Additional questions remaining are whether the differences induced by ribavirin are specific for HBV infection and whether the same takes place in for instance HCV infection.

The effect of type I IFN on antigen-presenting cells

When observing the IFN-based therapy in our study in chapter 3 one will notice that the most profound therapy-induced effects found were not caused by ribavirin, but by peginterferon. Despite that the activity of type I IFN was originally described

close to 60 years ago [14], and the use of IFN as antivirals in hepatitis B infection stems from around 40 years ago [15], we still have only a limited understanding of their effects in antiviral immunity. IFN treatment is known to cause decreased blood counts in a dose-dependent fashion [16]. Indeed, in our study peginterferon treatment of chronic HBV patients induced a strong decrease in absolute leukocyte numbers. These haematological changes are mostly considered side-effects, of which anaemia, thrombocytopenia and leukopenia are the most prominent and can be attributed to a direct effect of IFNα on bone marrow [16]. The role that IFN play in inducing an antiviral state can roughly be divided into (a) the induction of IFN response genes and (b) the modulation of immune cells. In our study in chapter 3, also the relative numbers decreased for mDC (both BDCA-1$^+$ and BDCA-3$^+$) and monocytes, which may be due to the immune modulatory effects of IFN. It was noted earlier that the fact that leukocyte numbers decline shortly after starting treatment and recover within days to baseline levels after stopping treatment, suggests that a redistribution of leukocytes takes place from the peripheral circulation to other compartments like tissues [16]. In our study the decline in cell numbers starts within 12 weeks, possibly much earlier, while cell numbers return to baseline after discontinuing treatment. Although we did not see changes in adhesion markers on DC upon treatment of chronic HBV patients, there were changes in chemokine receptors. This suggests a different migration pattern that may cause a redistribution of these leukocytes. Moreover, it could be that the sub-populations of leukocytes that actually migrated to the liver show an even more changed phenotypical profile. Namely, a profile that leads to actual migration and perhaps also functional change. In agreement with this data, earlier reports show that IFNα *in vitro* and *in vivo* alters chemokine receptor expression [17, 18]. Further immune modulatory effects of type I IFN therapy are on proliferation, differentiation, activation and maturation of not only DC, but also various other leukocyte populations like monocytes, macrophages and lymphocytes [19]. Monocytes and mDC incubated with IFNα obtained an increased maturation status [20]. We were able to show a similar IFN therapy-induced increase in maturation status of DC and monocytes measured by markers like CD40, CD80, CD86. Specifically the CD14$^+$ CD16$^-$ monocytes and not the CD14$^-$ CD16$^+$ monocytes showed this increased maturation (data not shown). However, we could not show that these cells were endowed with higher functional potential, leading for instance to a higher capacity to induce T cell activation [19]. Since we performed T-cell stimulatory assays with total PBMC, the relative decrease of mDC – the most potent of antigen-presenters – may be to blame for this. In addition to what we reported in our paper, that focuses mostly on DC, in our study we also found IFN effects on other immune cells: the numbers of peripheral NK cells strongly increased, caused primarily by an increase in CD56^{bright} NK cells. Increased CD56^{bright} NK cell numbers during peginterferon treatment of chronic HBeAg-negative HBV patients were reported to be accompanied by a concomitant enhanced expression of activation markers, activating receptor NKp46, TRAIL and IFNγ [21]. A similar effect was found in the treatment of chronic HCV patients with IFN-based therapy. This led to an increased frequency of CD56^{bright} NK cells, while not only the CD56^{dim} NK cell frequency decreased but also perforin and CD16 levels strongly dropped [22]. It has been suggested that this shift in NK cell subsets may result in more NK cells able to produce IFNγ while these cells demonstrate less cytotoxic capacity possibly resulting in less liver damage [22]. However, since these studies were performed on peripheral cells, the effects of these peripheral cells on the intrahepatic immune system are unsure. Moreover, it could be that the CD56^{dim} to CD56^{bright} shift took place because of migration of NK cells to the liver. As with ribavirin, perhaps

more interesting would be the effect of IFN on intrahepatic immune cells. It was reported that during IFN treatment intralobular CD68 expression (denoting KC) was stable. However, intrahepatic CD14 expression was increased, likely representing monocytes/infiltrating macrophages [23], while also NK cell numbers were increased in the portal areas [24]. Together these cells presumably reflect an IFNα-induced enhancement of the antiviral immune response. CD1a and CD83 expression was unaltered by IFN therapy, while there are contrasting reports on HLA-DR expression [23, 24]. At the same time, T-cell infiltration was reduced, mostly caused by a decrease in $CD8^+$ T cell numbers [23]. Further assessment of IFN-induced changes and what functional consequences such changes would have remain to be performed.

Ultimately, the goal of IFN therapy is helping the patient, who did not spontaneously clear the virus, to get rid of the virus after all. In our study only 4 out of 14 patients presented a combined virological and biochemical response, while the majority failed to do so despite the immunological changes induced by IFN and ribavirin. None of these changes in phenotype or function correlated with response to therapy, suggesting that these changes found in peripheral immune cells do not aid or harm anti-viral immunity. Which immunological changes, peripheral but also intrahepatic, during treatment are of importance for clinical outcome remains to be assessed further. Not only for prediction purposes, like pre-treatment CXCL9 and CXCL10 can predict peginterferon treatment outcome [25, 26], but also for immunological understanding.

The effect of type III IFN on antigen-presenting cells

As mentioned in the introduction of this thesis, IFNλ is a type III IFN that induces ISG expression largely overlapping with that induced by IFNα. However, because of a more restricted expression pattern of its receptor compared to that of IFNα, IFNλ induces ISG only in specific cell types. RNA expression for IL-28Rα has been shown in blood and in most tissues, among which was liver [27, 28]. But as stated, only few cell types react to IFNλ. Peripheral blood monocytes, B cells, T cells and NK cells have been reported not to react to IFNλ [28], although on NK cells there are studies that conflict with this notion [29-31] as discussed in chapter 4. Within blood, only pDC have convincingly been shown to express the IFNλR and react to IFNλ [32], as have the *in vitro*-cultured macrophages that we look at in chapter 4 [33]. In our study we wanted to assess whether IFNλ can affect cells other than those that become infected, like hepatocytes in the viral hepatitis setting. Due to the lack of expression of the IFNλR1 subunit and the lack of reaction to IFNλ by NK cells, an interesting and important cell to combat HBV infection, we focused on IFNλ effects on macrophages. Functional interactions between NK cells and other innate immune cells like DC and macrophages are well described [34-38]. We then reported an indirect effect of IFNλ on NK cells, via macrophages (Figure 1A), a cell type that expressed the IFNλR and functionally reacted to IFNλ exposure. But monocytes, the precursor of our *in vitro*-generated macrophages, were negative for the IFNλR1 subunit. Exposing monocytes to GM-CSF over time caused upregulation of IFNλR1, showing the effect of environmental factors on the expression of this receptor. Since the liver has a cytokine milieu different from peripheral blood in steady state conditions, but also in inflammatory conditions like during HBV infection, it would be interesting to see what the expression of the IFNλR on immune cells in the liver is. Interestingly, *in vitro*, murine peritoneal and bone marrow-derived macrophages

were shown not to react to IFNλ, suggesting a lack of functional IFNλ receptor on tissue and another type of *in vitro*-generated macrophages [39, 40]. Moreover, KC from healthy human liver showed no IFNλR1 expression and cytokine production did not change upon exposure to IFNλ (data not shown). The cause of this discrepancy in IFNλR expression between KC and monocyte-derived macrophages may be in the liver environment, but could also be explained by possible differences in origin [41]. Phenotypically, M1-like macrophages like the monocyte-derived macrophages that we cultured differ from KC (data not shown), which is likely caused by the liver environment. It remains to be seen whether infiltrating macrophages also express IFNλR within the liver. If they do, this suggests that infiltrating monocytes/macrophages are the cells that react to IFNλ, not KC, producing cytokines that induce NK cell function, but perhaps also adaptive immunity. Whether this indeed takes place during hepatitis B infection, in an altered hepatic cytokine milieu and in the presence of HBV and its proteins that on the one hand inhibit IFN signalling, but on the other hand induce cytokine production by macrophages (Figure 1B), remains to be investigated (see Figure 1C).

Taken together, based on literature and our data in chapter 3, it seems most likely that ribavirin enhances the IFN response instead of employing any of the other described methods. Since IFN in our study induced a proper clinical response in only a minority of patients despite the IFN-induced immunological changes, it is tempting to conclude that the antiviral capacity of IFN is mostly targeted at hepatocytes. However, although HBV inhibits IFN signalling in human hepatocytes [42], during acute HBV infection of chimpanzees no IFN signalling was found in liver tissue as a whole, including immune cells [43]. Additional factors like increased bile acid concentrations during HBV infection are able to disturb IFN signalling in not only hepatocytes but also immune cells [44]. Therefore, we can certainly not exclude that the therapeutic effect of IFN is accomplished via not only a direct effect on hepatocytes but also an immune-modulatory effect making use of APC. Furthermore, since KC and peripheral immune cells, except pDC, lack the functional IFNλR it is hard to determine the importance of IFNλ in the local immune response. It seems unlikely that IFNλ, inducing an ISG pattern largely overlapping with that of IFNα, would perform better than IFNα as a therapeutic in HBV infection, except for an improved tolerability. However, based on the observation that IFNλ induces an ISG response that is more sustained than that induced by IFNα, IFNλ may fit the requirements for a therapy that provides a long-lasting protective immune response [45, 46]. Additionally, the mechanisms inducing the expression of type I and type III IFN are not identical [47, 48], suggesting differential actions and/or similar actions differentially induced, perhaps as a resistance mechanism to viral evasion strategies. Moreover, due to its restricted receptor expression IFNλ may exert biological efficacy on other cell types than IFNα does, limiting its actions to certain cells. Therefore, until important questions like intrahepatic receptor expression, importance of infiltrating macrophages in the anti-viral immune response, possible unfavourable pro-viral IFNα effects that IFNλ might not induce and most importantly, clinical efficacy of IFNλ, have been addressed IFNλ will remain a promising therapeutic option for HBV infection worth exploring.

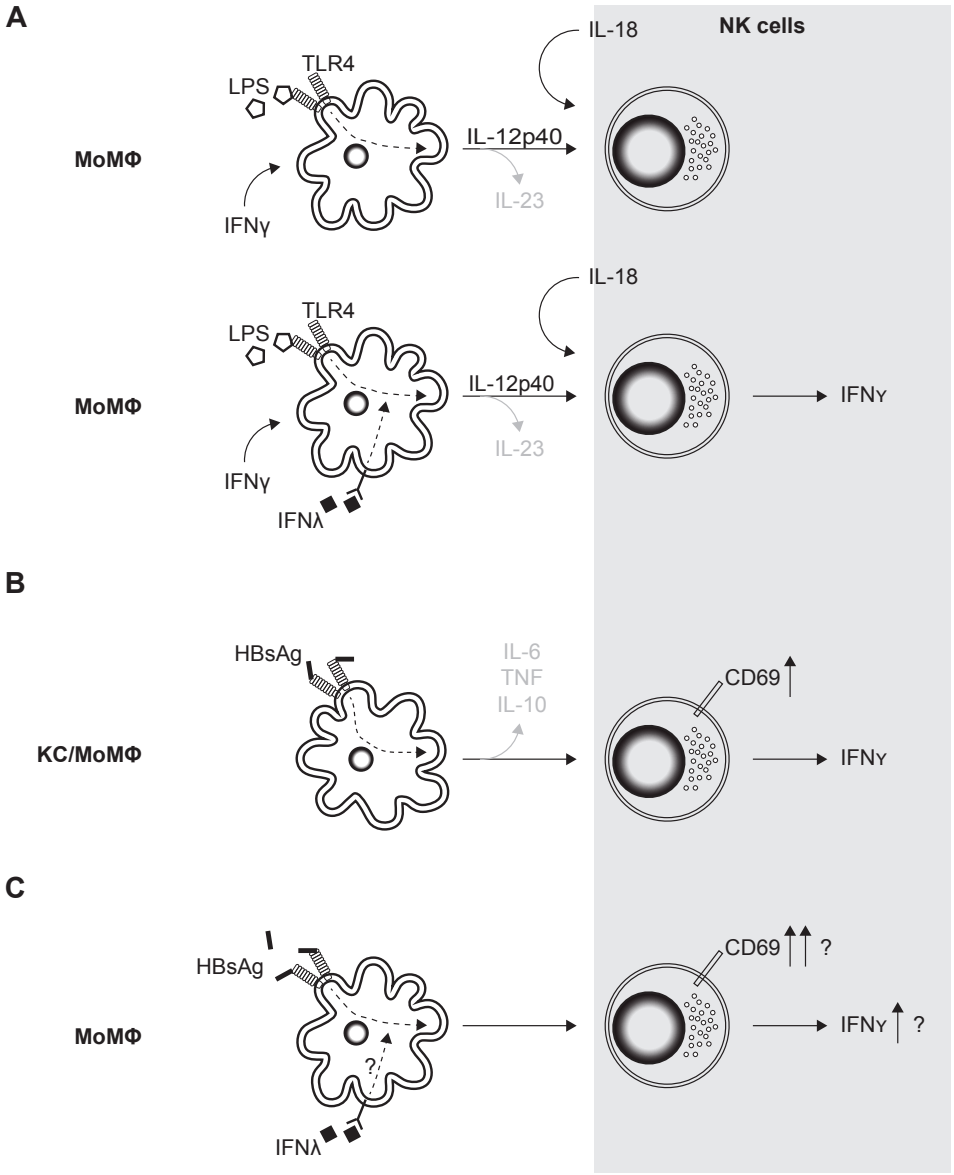


Figure 1. KC and monocyte-derived macrophages induce NK cell function. (**A**) Addition of IFNλ activates LPS-induced monocyte-derived macrophages to produce IL-12p40 and IL-23, and to induce (IL-12-dependent) IFNγ production by NK cells. (**B**) HBsAg-induced monocyte-derived macrophages and KC produce IL-6, TNF and IL-10, and induce IFNγ production by and activation of NK cells. (**C**) Will addition of IFNλ to HBsAg-induced monocyte-derived macrophages further induce IFNγ production by and activation of NK cells? KC, Kupffer cells; MoMΦ, monocyte-derived macrophages.

Part II Effect of HBsAg on function of antigen-presenting cells

The recognition of HBV

Even though recently an HBV receptor has been identified on hepatocytes [49], it remains to be resolved how immune cells recognize HBV and what the functional consequences of this interaction are. It has been stated that HBV is a stealth virus since, in contrast to HCV and other viruses, HBV does not seem to induce any type I IFN and ISG during the early stages of infection [43, 50]. And although it is difficult to assess such early events in acute HBV infection in humans, it was recently shown that type I IFN responses are also absent in acute HBV patients [51]. Moreover, it was shown that pDC, immune cells specifically known for their ability to produce copious amounts of IFNα upon recognizing viruses, display no IFNα response to HBV exposure [38]. However, despite not inducing the type I IFN response normally seen upon viral infection, over 90% of infected adult patients clear the virus spontaneously [52]. This indicates that a proper antiviral response is mounted nonetheless, meaning that HBV does not stay under the radar but is recognized, in which APC an important role. By recognizing HBV, APC can start and from there on regulate the antiviral immune response. It was already shown that non-parenchymal cells, presumably KC, react to HBV by secreting pro-inflammatory cytokines IL-1β, IL-6, TNF and CXCL8 [50]. Liver macrophages ($CD68^+$) and a $CD11c^+$ intrahepatic population (either macrophages or mDC) also produce IL-23 during HBV infection [53]. Monocytes produce TNF and IL-10 upon HBsAg exposure [54], while monocyte-derived DC produced IL-12 [55]. In a different study both HBsAg and HBcAg induce IL-23 production in monocyte-derived DC, but little IL-12 production [53]. However, besides stimulatory capabilities, it has also been reported that HBV and its components are inhibitory, being able to suppress TLR-induced cytokine production in various cell types [38, 56-61].

In chapter 5 and 6, we show that patient-derived HBsAg induces cytokine production in not only monocytes, but also in KC and monocyte-derived macrophages; cells that are found at the place of infection, i.e. the liver. Although we could not show the presence of HBsAg on or in monocytes, there are several findings that corroborate our HBsAg-induced monocyte data [54, 62, 63]. This would indicate not only an interaction but also the capacity to recognize HBsAg and act on it. Likewise, there has been circumstantial evidence suggesting the interaction of KC with HBV [50]. Although we show that both monocytes and KC interact with and react to HBsAg *in vitro* by producing cytokines, whether the latter takes place *in vivo* is unsure. In our study in chapter 5 we speculate that cytokine production by peripheral monocytes in chronic HBV patients is likely regulated. Also cytokine production in the liver might well be regulated, since the liver is known for its tolerogenic environment. If HBV and its proteins turn out to induce KC and monocytes *in vivo*, it is likely that KC, and not monocytes, are the cells that initiate an antiviral response since KC are already at the place where it is needed: in the liver. KC can produce chemokines and cytokines to attract and activate other immune cells. Monocytes can add to this response by removing viral proteins from the periphery and producing cytokines influencing peripheral immune cells. Monocytes can also infiltrate the liver and reinforce the antiviral inflammatory response by differentiating to macrophages. Due to the lack of distinctive markers further analysis of the role of KC versus liver-infiltrating macrophages is hampered. How either of these cells contributes to viral immunity

and disease pathogenesis should be assessed in future efforts.

As both monocytes and macrophages interact with HBsAg, an interesting and more often proposed putative receptor for HBV is CD14 [63]. CD14 was put forward as a possible receptor on peripheral monocytes for recombinant HBsAg, in combination with LPS-binding protein (LBP). However, the same paper showed that HBV patient plasma-derived HBsAg (pHBsAg) was not endowed with the capacity to bind to CD14 via LBP [63], although we show that pHBsAg is able to activate monocytes and therefore likely to bind to them. Since CD14 is only expressed by a limited number of cell types, suggesting it as a possible receptor for HBV/HBsAg imposes a restriction on the number of cells able to bind HBV/HBsAg. This theoretically either excludes CD14-negative cells being able to interact with HBV or makes it necessary to introduce an additional putative receptor to do so. Since it has been shown that CD14-negative cells like BDCA-1$^+$ mDC and monocyte-derived DC interact with HBsAg (Shi 2007, Op den Brouw 2008 Immunology), the former of the two options seems unwarranted. However, as shown for LPS triggering, CD14-negative cells like DC, but also endothelial, epithelial and smooth muscle cells, are not CD14 negative in an absolute sense. Besides as a membrane-bound form (bound via its GPI-anchor), CD14 also exists in a soluble form (sCD14) either as a shedded molecule (48-49 kDa) or a secreted molecule (55-56 kDa). Although the biological difference between these forms of different size for now remains unknown, it is clear that sCD14 plays an important role in the LPS-mediated activation of aforementioned CD14-negative cells [64]. The same may hold true for HBsAg. Indeed, rHBsAg bound sCD14 as it did mCD14 [63]. Since sCD14 (and LBP) are found in serum *in vivo*, but also in *in vitro*-used serum-containing media, HBsAg may bind to sCD14. sCD14 then can bind CD14-negative cells like DC, but also CD14-positive cells, via its GPI-anchor and hereby activate the cell. Since the interaction between sCD14, LPS and LBP is fundamentally different from that between mCD14, LPS and LBP [64], it could well be that the resulting signalling induced by sCD14 and HBsAg likewise differs from that induced by mCD14 and HBsAg, resulting in a different cellular reaction pattern depending on the receptor used and the cell the receptor binds to or is bound to.

Another possible receptor for HBsAg put forward is the mannose receptor (MR; CD206), expressed by DC [65] and KC [66], and to a lesser extent by monocytes [67]. mDC were shown to bind HBsAg via the MR. HBsAg$^+$ mDC were especially found within the liver. Interestingly, mDC MR expression and HBsAg are positivity correlated, suggesting that the MR-mediated interaction between mDC and HBsAg mostly takes place at the site of inflammation, i.e. the liver. Additionally, HBsAg-induced IL-23 production by monocyte-derived DC and monocyte-derived Mf (MoMΦ) was shown to be dependent on the binding of HBsAg to the MR and subsequent endocytosis [53]. Interestingly, on cells that express both CD14 and CD206, co-ligation of these receptors can lead to a different response than ligation of the separate receptors [68]. This would indicate that also the balance of CD14 and CD206 expression levels may attune the reaction to their ligands per cell type. Accordingly, MR can induce production of cytokines like IL-10, IL-17, TNF, CCL2 and CXCL8 in response to some fungal pathogens, but paradoxically also inhibit the production of cytokines such as IL-12 and TNF in response to other fungal pathogens [53]. This adds another variable to the various reaction patterns of the different APC to HBsAg.

Consequence of APC interacting with HBV, and their role in the HBV-induced immune response

A. Self-limiting hepatitis B infection

As stated in the introduction, a coordinate activation of innate and adaptive immunity is key in controlling hepatitis B infection, with a central role for antigen-presenting cells. By recognizing HBV and its components, and subsequently secreting pro-inflammatory cytokines, KC are able to contribute to the immune activation and antiviral immunity upon HBV infection, as described in chapter 2. By secreting chemokines and cytokines, KC will not only attract and activate HBV-specific immune cells like $CD8^+$ T cells. Also non-specific immune cells like monocytes, DC and NK cells will be attracted. Monocytes, like we show in chapter 5, in turn can react to HBV and/or its components by producing cytokines. Moreover, monocytes can differentiate into macrophages upon entering the liver, becoming infiltrating monocyte-derived macrophages. In chapter 6 we showed that monocyte-derived macrophages can bind HBsAg, subsequently becoming activated and producing pro-inflammatory cytokines. We showed that both HBsAg-induced KC and monocyte-derived macrophages were able to induce NK cells to produce IFNγ production. Additionally, monocyte-derived DC derived from $HBsAg^+$ monocytes from the peripheral blood of chronic HBV patients were shown to cross-present intracellularly stored viral antigen to autologous HBV-specific T cells, inducing IFNγ secretion [62]. Among the APC, DC are known to be excellent cytokine producers and the most efficient antigen presenters, and therefore are thought to play a central role in viral infections like HBV infection [3, 69]. Despite that DC have been shown to be functionally impaired in chronic HBV patients [58, 70-72], there is a direct interaction between DC and HBV [65]. In acute HBV infection there may hence still be a pivotal role for these cells.

Together, APC will be able to regulate antiviral immunity, bridging innate and adaptive responses, leading to a self-limiting infection in most cases among adults.

B. Chronic hepatitis B infection

The abundant cytokine production by monocytes from healthy individuals upon *in vitro* exposure to HBsAg suggests that *in vivo* a constant production of cytokines takes place, since peripheral blood monocytes are constantly exposed to high levels of HBsAg. However, chronic hepatitis B patients have not been reported to have exceedingly high levels of cytokines in their blood, suggesting that some regulatory mechanism is in place that restrains the monocytes' reaction to viral proteins. A mechanism like sensitization, due to continuous exposure to a certain stimulus, seemed a logical explanation given the high levels of viral particles in patients' blood. However, based on the unaffected cytokine production upon TLR ligand exposure, the variable concentrations of HBV DNA, HBeAg and HBsAg did not affect the ex vivo functional inducibility of monocytes. What determines the differential effects that have been reported for viral proteins, being either inhibitory [38, 56, 58, 60, 73-77] or activatory [50, 53-55] and [this thesis] remains to be assessed. As put forward in our study, regulatory cytokines like IL-10 and TGFβ [51, 78-81], are molecules that have inhibitory effects on cytokine production *in vivo*. We showed that both molecules are

able to abrogate HBsAg-induced cytokine production *in vitro*. An additional factor in the regulation of the monocyte response to HBsAg may be sCD14, based on a similar function this molecule has in LPS-induced activation of monocytes and clearance of apoptotic bodies [64, 82]. sCD14 binds and clears apoptotic bodies without cellular activation and pro-inflammatory cytokine secretion [64, 82]. Besides enabling CD14-negative cells to react to LPS or HBsAg, sCD14 may also inhibit the reaction of mCD14-positive cells by competition for the binding of the ligand [64]. Indeed, adding sCD14 to monocyte cultures strongly decreased binding of HBsAg to the monocytes [63]. After stimulation, monocytes secrete sCD14 [83]. In chronic hepatitis B patients, blood sCD14 levels were higher than those in acute hepatitis B patients and healthy controls. Moreover, sCD14 levels inversely correlated with the HBsAg levels in chronic HBV patients, but not with HBV-DNA levels [82]. This suggests that HBsAg influences sCD14 levels, either by decreasing secretion or by increasing removal from the circulation. Since we performed our monocyte studies in X-VIVO medium, without any serum and thus sCD14, it is interesting to see what the addition of serum and/or sCD14 does to the monocyte-HBsAg interaction in our cultures, and what exactly takes place mechanistically.

Especially cirrhotic chronic HBV patients showed high sCD14 levels [82]. Since also hepatocytes are known to express and secrete CD14 [64], it would be interesting to see how sCD14 levels are in the liver, and what the effect of these levels would be on intrahepatic immune cells like KC.

Although the cytokines that monocytes and KC produce upon HBsAg exposure separately have been shown to inhibit HBV replication [50, 84], in combination with each other, with additional cytokines and with other soluble molecules the effect becomes more complex as we discussed in chapter 2 and describe below. Recently, it has been shown that the sodium/bile acid co-transporter Na^+-taurocholate cotransporting polypeptide (NTCP), expressed mainly by hepatocytes, is a receptor for HBV. This receptor interacts with the pre-S1 of HBsAg L. Using siRNA to reduce NTCP expression, it was possible to inhibit HBV infection in the HepaRG cell line [49]. NTCP expression is reduced by LPS via the reduction of the RXRα/RARα heterodimer. IL-1β turns out to play a major role in this, causing the export of RXR from the nucleus. Further reduction of NTCP is caused by a reduction of transcription factors like HNF1α. Besides IL-1β, also IL-6 and TNF decrease NTCP expression [85]. IL-6 has been shown to inhibit NTCP expression via the reduction of HNF1α [86]. Additionally, IL-6-induced inhibition of HNF1α, a transcription factor essential for HBV gene expression and replication, led to the inhibition of HBV. Since KC are in the proximity of hepatocytes, we hypothesised that cytokines produced by HBsAg-activated KC, especially IL-1β, IL-6, and TNF, separately but also together would inhibit HBV replication. To test this we set up experiments using the HepaRG cell line. We differentiated these cells and infected them with HBV, in the absence or presence of single cytokines or conditioned medium containing KC supernatants. We harvested the hepatocytes and measured the HBV total RNA and HBV 3.5 kb RNA for HBV pre-C and pregenome RNA (pgRNA) by PCR. Following infection of differentiated HepaRG cells with HBV we were able to measure HBV 3.5 kb RNA (Figure 2A) and HBV total RNA (data not shown), indicating active HBV replication. In the same figure we show that undifferentiated HepaRG did not support HBV infection. In accordance with literature, adding IL-6 or IFNγ to the culture inhibited HBV replication. However, the addition of IL-1β and TNF increased HBV replication (Figure 2A). Besides single cytokines, we also cultured infected HepaRG cells in conditioned medium, as shown in Figure 2B. This conditioned medium contained

supernatant of unstimulated (medium condition) or HBsAg-stimulated (HBsAg condition) KC. Interestingly, when incubating infected HepaRG cells with conditioned medium HBV replication is increased, as indicated by an increase of HBV 3.5 kb RNA (Figure 2B) and HBV total RNA (data not shown). Conditioned medium containing unstimulated supernatant induced an increase of HBV replication similar to that induced by IL-1β or TNF, while conditioned medium containing HBsAg-stimulated supernatant induced HBV replication even 2 to 4 times more potently. These data show that KC do not necessarily have an inhibitory effect on HBV, despite producing cytokines that are known to inhibit HBV replication (Figure 3A). Apparently, combinations of cytokines that we measured and/or other cytokines and soluble molecules in this case induce HBV replication in an infected HepaRG cell line (Figure 3B). Moreover, the notion that unstimulated KC, albeit to a lesser extent than stimulated KC, induce HBV replication suggest that KC constitutively produce pro-viral factors that increase when they are activated. It remains to be investigated what factors play a role in increasing KC-induced HBV replication. In addition to the example above, in chapter 2 we described that KC can play not only an important role in building up anti-viral immunity against HBV, but also in tissue damage and development of viral persistence.

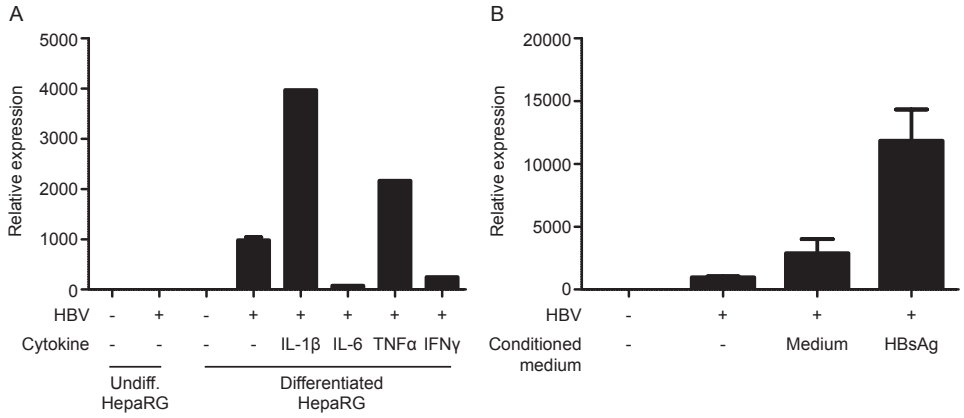


Figure 2. HBsAg-induced KC supernatants enhance HBV replication. HepaRG cells were incubated with medium or HBV for 16 hours in the presence or absence of cytokines (A) or KC supernatant (B). Subsequently HepaRG cells were washed and cultured further for 10 days in the presence or absence of the same cytokines (A) or KC supernatant (B), partly refreshing medium, cytokines and/or supernatants every 2 days. 10 days after infection relative expression of HBV 3.5 kb RNA was measured by PCR. Undiff, undifferentiated.

Together, APC play a role in chronic HBV infection: on the one hand failing to build up an effective innate and adaptive immune response, and on the other hand causing tissue damage and aiding in the development of fibrosis, cirrhosis and HCC.

Concluding remarks

This thesis focused on the role of APC in HBV infection, both in the peripheral blood and in the liver. DC, monocytes and KC all interact with HBV and its proteins. In

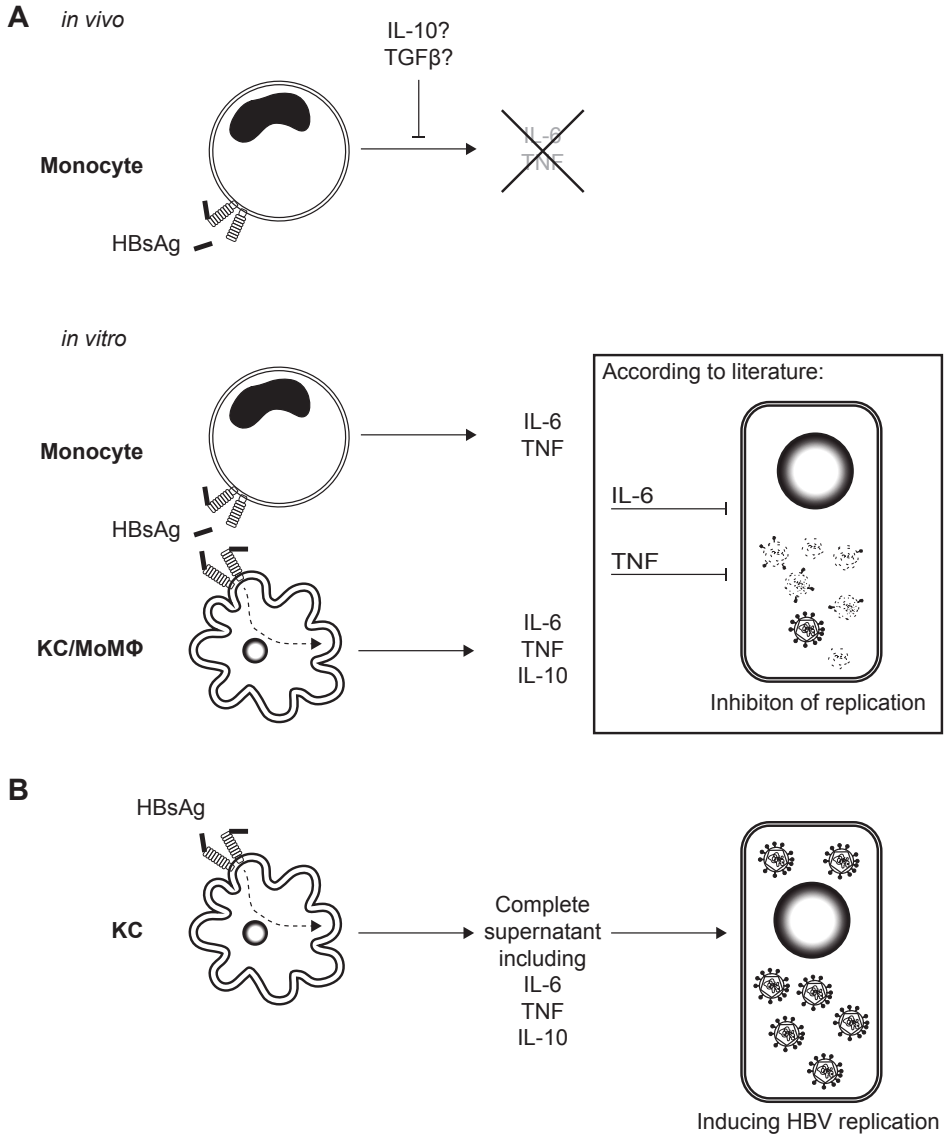


Figure 3. HBsAg-induced KC cytokines enhance HBV replication. (**A**) While chronic *in vivo* exposure of peripheral monocytes to HBsAg does not lead to high cytokine levels, possibly due to regulation by molecules like IL-10 and TGFβ, *in vitro* exposure of monocytes, KC and monocyte-derived macrophages leads to high expression of cytokines like IL-6, TNF and IL-10. IL-6 and TNF have been shown in literature to be able to inhibit replication of HBV. (**B**) Supernatants of medium-induced (not shown) and HBsAg-induced KC, including the cytokines IL-6, TNF and IL-10, in our experiment did not lead to inhibition of HBV replication, but enhancement of HBV replication.

chapter 6 we showed a direct interaction between KC and HBsAg, *in vivo* and *in vitro*. Chapters 5 and 6 combined clearly show that *in vitro* HBsAg strongly induces monocytes, monocyte-derived macrophages and KC to produce pro-inflammatory cytokines. HBsAg-activated KC and monocyte-derived macrophages subsequently induced NK cell activation and IFNγ production. Cytokines like IL-6 and IFNγ have been shown to have direct antiviral effect. Together, this suggests that

KC, but perhaps later also inflammatory monocytes play an important role in the initiation of the antiviral immune response. Additionally, in chapter 4 we showed that IFNλ, a possible future HBV therapeutic option, potentiates IL-12 secretion by monocyte-derived macrophages enhancing their NK-cell stimulatory capacity. Since we showed that KC do not express the IFNλR, this might signify a role for liver-infiltrating monocyte-derived macrophages in IFNλ-induced antiviral immunity, enhancing their IL-12 family cytokine production that can activate NK cells but has also been shown to be important for T cells. Another IFN, IFNα, one of the first-line therapeutics in HBV infection was shown to directly activate NK cells. The ability of these IFN to activate NK cells seems redundant, but it may also signify a difference in antiviral mechanism where IFNα induces a short, strong antiviral reaction, while IFNλ induces a slow and long-lasting protective immune response. Furthermore, as shown in chapter 3, IFNα also induced an increase in activation status in mDC and pDC, an effect that was slightly enhanced by the addition of ribavirin. Although in our study these immunological changes did not lead to an improved clinical outcome, they may improve the induction of antiviral immunity and/or correlate with clinical outcome. Possibly, by improving the IFN response in HBV patients, immunological changes might be more profound and be able to tip the balance towards an efficient antiviral immune response.

In contrast to activatory effects, in chapter 6 HBsAg was also able to induce IL-10 secretion by KC and monocyte-derived macrophages, suggesting an additional immune regulatory role for these cells. Also, despite the induction of pro-inflammatory cytokines in monocytes *in vitro*, constant exposure to serum viral components *in vivo* did not alter monocyte function, suggesting that also here regulatory mechanisms may be at play. Furthermore, as shown in chapter 7, when incubating infected hepatocyte cell lines with supernatants from KC, either unactivated or activated by HBsAg, HBV replication was promoted, despite the presence of TNF and IL-6 in these supernatants. As discussed in chapter 2, these are not the only deleterious effects APC may have.

Together, this shows that APC can have opposing roles. On the one hand they may play a pivotal role in initiating the immune response against HBV. On the other hand they can also disturb the immune response and support viral replication. Factors like the dual effects (inhibitory/activatory) that HBV proteins can have, but also the dual effects (antiviral/virus-promoting) that antiviral cytokines can have will contribute to this. What determines what exact effects these cytokines and viral proteins have on APC, on HBV, on the liver remains to be determined, especially when they are combined.

Future HBV research will require a systematic analysis, intrahepatically and peripherally, of the role of specific types and subsets of APC involved in hepatitis B infection. Not only in chronic HBV, but also in acute HBV, in order to define which APC features like phagocytosis, cytokine production or NK cell activation determine resolution of the infection, be it spontaneously or therapy-induced, and what APC features determine advancement towards chronicity despite therapy.

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Chapter 8

Nederlandse samenvatting voor niet-ingewijden

Algemene introductie

In het kort: Wat is hepatitis, wat zijn de spelers in de immuunafweer en waar is mijn onderzoek op gericht?

Hepatitis B virus

Hepatitis B virus (HBV) is een virus dat specifiek de lever infecteert. Deze infectie resulteert in acute en daaropvolgend mogelijk chronische leverinfectie, ofwel hepatitis. HBV wordt overgedragen van moeder op kind tijdens de geboorte, via bloed-bloed contact, bijvoorbeeld via een wond in de huid of een naald, of via onbeschermd seksueel contact. Infectie met HBV vindt vaak zonder ziekteverschijnselen plaats. Als die er toch zijn dan is de patiënt vaak geel (huid, oogwit), moe, misselijk en plast bijvoorbeeld donkere urine. Het risico op het ontwikkelen van een chronische infectie hangt af van de leeftijd van de patiënt, maar ook van de infectieroute en de dosis (hoeveelheid virus die binnendringt). Tot wel 90% van de HBV-geïnfecteerde pasgeborenen ontwikkelt chronische hepatitis. Het merendeel van de geïnfecteerde volwassenen daarentegen klaart het virus uit zichzelf, terwijl slechts een klein deel dit niet lukt en een chronische vorm van hepatitis ontwikkelt. Momenteel zijn meer dan 240 miljoen mensen wereldwijd chronisch geïnfecteerd met HBV. Dit kan leiden tot voortschrijdende leverschade waardoor de patiënt een verhoogd risico heeft op het ontwikkelen van levercirrose (verlittekening van de lever), leverfalen en leverkanker. De gevolgen van HBV infectie leiden tot de dood van ongeveer 600.000 mensen per jaar. Het is kortom een belangrijk wereldwijd gezondheidsprobleem.

Het virusdeeltje ziet er in het kort als volgt uit (zie figuur 2 in hoofdstuk 1): het DNA van het virus (viraal DNA) wordt omgeven door een kapsel. Dit kapsel bestaat uit een grote hoeveelheid exemplaren van een eiwit dat we HBV kapsel antigeen (“HBV core antigen”, HBcAg) noemen. Om het kapsel heen zit een envelop, die weer uit een grote hoeveelheid exemplaren van het eiwit HBV oppervlak antigeen (“HBV surface antigen”, HBsAg) bestaat. In een geïnfecteerde cel wordt het DNA gebruikt om HBcAg, HBsAg en andere eiwitten te maken, maar ook nieuwe HBV DNA. Vervolgens wordt het virusdeeltje opgebouwd. Virusdeeltjes en onder andere HBsAg worden in grote getalen aangemaakt en kunnen beide in het bloed van patiënten gemeten worden.

Immuunrespons

Wanneer het lichaam blootgesteld wordt aan een virus vormt het zogenaamde aangeboren (“innate”) immuunsysteem de eerste barrière tegen binnendringende ziekteverwekkers, ook wel pathogenen genoemd. Het aangeboren immuunsysteem bestaat uit verschillende onderdelen, waaronder fysieke en chemische barrières zoals de huid en slijmvliezen, maar ook immuuncellen waarvan een aantal in dit proefschrift besproken worden. Voorbeelden van ‘innate’ immuuncellen zijn NK (“natural killer”) cellen en de groep cellen die we fagocyten noemen. Dit zijn cellen die gespecialiseerd zijn in het opeten van onder meer schadelijke deeltjes, bacteriën en virussen. Fagocyten zoals monocyten, macrofagen en dendritische cellen (DC) struinen de omgeving af op zoek naar virus om op te eten en vervolgens te vernietigen, waardoor ze de hoeveelheid virus in het lichaam verminderen. In plaats van het

virus te vernietigen kunnen ze het ook verwerken tot kleine stukjes virus (antigeen) die ze aan andere cellen van het aangeleerde (“adaptive”) immuunsysteem kunnen laten zien; dit noemen we antigeenpresentatie. Vanwege deze functie worden monocyten, macrofagen en vooral DC professionele antigeen-presenterende cellen (APC) genoemd. De cellen van het aangeleerde immuunsysteem, B en T cellen, worden hierdoor geactiveerd zodat ze het virus en virus-geïnfecteerde cellen kunnen aanvallen. Zie figuur 1 in hoofdstuk 1 voor een schematische weergave van de rol van APC in de antivirale immuunrespons.

Interferonen

Belangrijke spelers in het starten en reguleren van de respons tegen virussen in het algemeen, de zogenaamde antivirale response, zijn interferonen (IFN). Deze moleculen hebben niet alleen een directe antivirale werking door allerlei mechanismes aan te zetten in geïnfecteerde cellen en hun buurcellen, maar hebben ook indirect effect op virussen door immuuncellen te reguleren.

In dit proefschrift kijken we naar twee types interferonen: de type I en de type III interferonen. De twee type I interferonen, IFNα en IFNβ, worden tijdens een virusinfectie gemaakt door allerlei cellen (bijvoorbeeld geïnfecteerde cellen maar ook immuuncellen). Vooral bepaalde DC, de plasmacytoide DC (pDC), staan bekend om hun grote vermogen om type I IFN te maken. De meeste cellen reageren op type I IFN, omdat ze op hun oppervlak een receptor voor IFN dragen. Type I IFN is uiteindelijk in staat om virussen, waaronder HBV, direct te remmen, maar ook om immuuncellen te activeren. Echter, tijdens HBV infectie wordt er geen type I IFN gemaakt. Het kan wel van buitenaf gegeven worden, als medicijn, wat kan leiden tot het verminderen van de HBV infectie, in een deel van de patiënten zelfs tot klaring van het virus (genezing). Type III interferonen, de IFNλ, zijn recent ontdekte interferonen. Ze worden door veel minder cellen geproduceerd, maar hebben ook op veel minder cellen effect omdat veel minder cellen de receptor voor deze interferonen dragen. Ook deze interferonen hebben echter de capaciteit om virussen waaronder ook HBV direct te remmen en immuuncellen te activeren.

Immuuncellen

Het in dit proefschrift beschreven onderzoek is gericht op de hierboven genoemde professionele antigeen-presenterende cellen. Vooral de myeloide DC (mDC) zijn efficiënt in het opnemen, verwerken en presenteren van antigeen. DC hebben als speciale eigenschap dat ze tijdens een infectie, na het opeten van bijvoorbeeld een virus, naar lymfeklieren kunnen gaan om daar de immuunrespons op gang te brengen door T en B cellen te activeren. Ook kunnen zij, samen met pDC, monocyten en macrofagen, stoffen maken die immuuncellen aantrekken en activeren. Deze stoffen noemen we cytokines. Macrofagen staan vooral bekend om hun hoge capaciteit om ziektekiemen als virussen op te eten en mogelijk te herkennen. Het is belangrijk om niet alleen naar immuuncellen in het bloed te kijken, maar ook naar immuuncellen in het weefsel dat geïnfecteerd is, in dit geval de lever. Daarom hebben we macrofagen onderzocht die je specifiek in de lever kunt vinden. Deze levermacrofagen heten Kupffer cellen. Daarnaast hebben we gekeken naar macrofagen die we vanuit

monocyten gemaakt hebben.

De onderzoeksvragen

In dit proefschrift onderzochten we twee aspecten van de rol van APC tijdens infectie met HBV: ten eerste hebben we gekeken naar de effecten van IFN op de functie en het uiterlijk van APC (hoofdstuk 3 en 4); ten tweede hebben we gekeken naar de interactie tussen APC en virale eiwitten, in het bijzonder die tussen APC en HBsAg (hoofdstukken 5 en 6).

Dit proefschrift richt zich op aspecten van de rol van APC bij chronische HBV-infectie. Verschillende studies benadrukken het belang van APC in het op gang brengen en reguleren van de antivirale immuunrespons. Daarom gaan we er van uit dat APC in het algemeen een centrale rol spelen in de antivirale immuunrespons tegen HBV. Echter, wat de rol van elk type APC is in HBV-infectie dient nog nader te worden gespecificeerd. IFN worden gebruikt als antivirale therapie bij chronische HBV-infectie, maar wat de precieze effecten van IFN op APC zijn en hoe dit invloed heeft op de rol van APC tijdens HBV infectie blijft onduidelijk. Daarnaast zijn APC voortdurend blootgesteld aan virale eiwitten, maar de literatuur waarin een duidelijke interactie tussen deze virale eiwitten en APC aangetoond wordt is schaars, terwijl literatuur over de effecten van virale eiwitten op APC tegenstrijdig en/of onvoldoende is. Daarom is ons doel de effecten van IFN en virale eiwitten op APC tijdens chronische HBV infectie te onderzoeken en zo de rol van APC tijdens HBV infectie verder in te kunnen vullen.

Deel I. Effecten van IFN op de functie en fenotype van antigeen presenterende cellen

Na hoofdstuk 2, waar we in een literatuuroverzicht de rol beschrijven die KC spelen en kunnen spelen bij chronische hepatitis B en C infecties, wordt in deel I (hoofdstuk 3 en 4) een aantal effecten van *in vivo* en *in vitro* IFN op APC aangesneden. In hoofdstuk 3 worden de effecten van de behandeling van chronische HBV patiënten met IFN monotherapie versus IFN/ribavirine combinatietherapie aan de hand van verschillende parameters bestudeerd. In hoofdstuk 4, worden de immunologische effecten van het nieuwe type III IFN *in vitro* op NK-cellen en macrofagen bestudeerd.

In het kort de belangrijkste resultaten

In hoofdstuk 3 laten we zien dat het toevoegen van het medicijn ribavirine aan de voor HBV gebruikte interferon therapie leidt tot dendritische cellen die meer geactiveerd zijn. Dit zou kunnen leiden tot een betere werking van het immuunsysteem tegen HBV en daaropvolgend meer patiënten die het virus klaren. Klinisch bleek dit echter niet het geval. Hoe dat komt is voer voor toekomstig onderzoek. In hoofdstuk 4 laten we zien dat het interferon type III IFNλ1, in tegenstelling tot het interferon type I IFNα, geen rechtstreeks effect op NK cellen heeft. Via macrofagen kan IFNλ1 alsnog effect uitoefenen op deze cellen. Dit indirecte effect van IFNλ1 op NK cellen vindt plaats

via de productie van een belangrijk pro-inflammatoir cytokine door macrofagen, namelijk IL-12. Verder onderzoek naar de antivirale en immuunregulatoire werking van IFNλ1 zijn van belang om te begrijpen hoe type III IFN vervolgens werken om bijvoorbeeld te bepalen of en waarom ze geschikt zijn als antivirale medicijnen.

Deel II: Interactie tussen antigeen-presenterende cellen en virale eiwitten, met name HBsAg

In hoofdstuk 5 en 6 onderzoeken we het effect van virale eiwitten op de immuunrespons. In hoofdstuk 5 bekijken we de gevolgen van blootstelling van monocyten aan virale eiwitten *in vivo* en HBsAg *in vitro*. In hoofdstuk 6 onderzoeken we KC van patiënten die chronisch geïnfecteerd zijn met HBV. We meten de aanwezigheid van HBsAg in deze cellen en hun uiterlijk. Daarnaast bepalen we het effect van HBsAg op het uiterlijk van de KC maar ook op dat van macrofagen die we vanuit monocyten gemaakt hebben.

In het kort de belangrijkste resultaten

In hoofdstuk 5 tonen we dat blootstelling van monocyten aan HBsAg leidt tot de hoge productie van cytokines; monocyten kunnen dus HBsAg binden en herkennen. Omdat monocyten in het bloed van HBV patiënten continu bloot staan aan virale eiwitten waaronder HBsAg zou je verwachten dat ook in het bloed hoge cytokinewaarden te meten zijn. Dit is echter niet het geval, wat suggereert dat in het lichaam regulatie plaats vindt van cytokineproductie van aan HBsAg-blootgestelde monocyten, bijvoorbeeld door een anti-inflammatoir cytokine als IL-10 of TGFβ. In hoofdstuk 6 laten we nogmaals zien dat HBsAg witte bloedcellen, in dit geval KC, aanzet tot cytokine productie. Maar in dit artikel laten we ook daadwerkelijk de directe interactie zien tussen KC en HBsAg die leidt tot cytokineproductie. Verder laten we zien dat HBsAg niet in staat is om NK cellen rechtstreeks te activeren, maar dit wederom kan doen via macrofagen, namelijk KC en macrofagen afkomstig van monocyten. De door HBsAg uitgelokte cytokineproductie en activatie van immuuncellen als macrofagen en NK cellen kan mogelijk een belangrijke eerste stap zijn in het opwekken van een immuunrespons tegen HBV na infectie.

Afsluitend: conclusies en resterende vragen

Dit proefschrift richtte zich op de rol van APC tijdens HBV infectie, zowel in het bloed als in de lever. In hoofdstuk 6 laten we een directe interactie zien tussen KC en HBsAg, en gezamenlijk laten hoofdstuk 5 en 6 duidelijk zijn dat monocyten, macrofagen afkomstig van monocyten en KC reageren op HBsAg door cytokines te produceren. Beide types macrofagen stimuleren vervolgens NK cellen tot de productie van IFNγ. Dit cytokine, samen met andere cytokines als IL-6 en TNF, hebben rechtstreeks antiviraal effect op HBV. Samen lijkt dit er op te wijzen dat KC, en mogelijk ook monocyten die naar de lever komen, een belangrijke rol spelen in het opzetten van een immuunrespons tegen HBV. Verder laten we in hoofdstuk 4 zien dat IFNλ1 de IL-12 productie van macrofagen verbetert waardoor ze beter NK cellen kunnen

activeren. Mogelijk kan IFNλ op HBsAg-gestimuleerde macrofagen en indirect op NK cellen eenzelfde effect hebben. IFNα, dat nu al gebruikt wordt als medicijn tegen HBV infectie, kan ook NK cellen activeren, maar dan rechtstreeks. Het bestaan van IFNα en IFNλ naast elkaar lijkt door overlappende mechanismes overtollig. IFNα heeft echter effect op veel meer cellen dan IFNλ, en bovendien lijkt IFNα een korter durend effect te hebben. Welke effecten van IFNα en IFNλ belangrijk zijn bij de opbouw van een antivirale respons en welke van de vele effecten niet nodig zijn of zelfs tegenwerken moet nog verder uitgezocht worden. Een celtype waar IFNα ook effect op heeft zijn DC zoals we laten zien in hoofdstuk 3. Maar ook patiënten die het virus niet klaren hebben door IFN-geactiveerde DC. Dit wijst er op dat het IFN effect op DC in onze studie onvoldoende is om klinisch het verschil te maken. Door de IFN respons verder te verbeteren in HBV patiënten kunnen er mogelijk grotere immunologische veranderingen teweeg gebracht worden door IFN therapie. Dit kan dan leiden tot het aanzetten van effectieve antivirale immuunrespons.

In hoofdstuk 7 laat ik uiteindelijk enige nieuwe data zien die tonen dat er nog een andere kant aan het verhaal kan zijn, namelijk het tegenwerken van de antivirale immuunrespons of het helpen van het virus. We laten in de andere hoofdstukken zien dat er cytokines geproduceerd worden waarvan bekend is dat ze antiviraal werken. Maar vaak is onbekend wat deze cytokines in combinatie doen. In de data in hoofdstuk 7 leek een combinatie van onder andere cytokines niet antiviraal te werken, maar eerder het virus te ondersteunen. Ook produceren KC en monocyten cytokines die juist de immuunrespons remmen, zoals IL-10, waarvan we in hoofdstuk 5 laten zien dat het ook de reactie op HBsAg kan remmen. Verdere effecten die bijvoorbeeld KC hebben die de antivirale respons niet helpen hebben we uitgebreid besproken in hoofdstuk 2.

Samen laat dit zien dat APC een tweeledige rol kunnen hebben. Aan de ene kant kunnen ze een centrale rol in de immuunrespons tegen HBV spelen. Aan de andere kant kunnen ze de afweer verstoren en het virus juist ondersteunen. Zowel virale eiwitten als cytokines beïnvloeden APC, maar wat bepaalt welk precieze effect deze hebben op APC en de immuunrespons in het algemeen en uiteindelijk op HBV moet nog nader bepaald worden. Hiervoor is verder onderzoek nodig, zowel in het bloed als in de lever, waarbij niet alleen APC in het algemeen, maar ook naar APC types en subsets gekeken wordt. De grote vraag blijft: welke eigenschappen leiden tot het klaren van het virus, en welke leiden tot chroniciteit (ondanks medicijnen)?

Dankwoord

Het is al weer even geleden dat ik deze reis aanving. Hoewel ik in de afgelopen jaren veel met de trein gereisd heb, heb ik de langste afstanden toch in mijn hoofd afgelegd. En ondanks dat dit werk een afsluiting is van een periode, voelt het slechts als een tussenstation, op weg naar een verdere bestemming die voor nu dichter bij huis ligt dan voorheen, maar toch ook verder weg. In dit deel van mijn proefschrift wil ik graag mijn medereizigers bedanken. Sommigen die net als ik, met weinig ervaring instapten en met wie ik een hoop ervaring heb opgedaan. Anderen waren reeds zeer bereisd waardoor ik een hoop heb geleerd tijdens mijn tocht. Zowel de eerste als de laatste groep, en een ieder die tussen deze twee groepen invalt, hebben het in meer of mindere mate mogelijk gemaakt deze reis te kunnen maken. Ik hoop dat, in welke categorie je ook denkt te vallen, de deelname aan mijn reis ook jullie verder heeft gebracht.

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Binnen mijn eigen treinstel: de virale hepatitis groep.

HBV: Marjolein, Paula, Gertine, Rekha, Lianne, Eric, Aniek, Evelyn, Nadine

HCV: Duygu, Anthonie, Dowty, Mark, Bi-Sheng, Rik, Kim, Michelle, Jun, Thomas

– waar te beginnen? Marjolein, mijn voorgangster – bedankt voor het laten zien hoe het moet. Rekha, hoewel het kort was, bedankt voor je hulp in het PARC project. Gertine, van wie ik me een oudere maar ook een jongere broer voelde – dat laatste als me weer eens verteld werd hoe het precies zat. Met jou had ik veel meer willen samenwerken, maar toch is het er nauwelijks van gekomen. Gelukkig was je er wel altijd! Lianne, de vreemde eend in de bijt: moleculaire biologie! Van jou heb ik veel geleerd. Aniek, Lianne's rechterhand, weinig mee samengewerkt, maar fijn om samen mee te werken. Eric, de man met mooie vragen over het leven en wat dies meer zij. En Evelyn en Michelle, de jonge oio's – "hoezo van onze leeftijd?" zei Evelyn hard lachend – bedankt voor de leuke tijd samen in het lab en er buiten. Anthonie, oude brombeer: met jou gingen die projecten toch veel sneller. Bedankt! Mark, altijd met flair. Dowty, één van mijn medereizigers die het zelfde traject aanging, maar dan via een andere route. Het was een plezier om met je op te trekken en nu, twee uur na aanvang van mijn verdediging is het jouw beurt. Succes! Jun, het gezelschap in het weekend, bedankt voor je werk aan het IFN-lambda artikel. En Thomas, voor het eerst echt gesproken op de liver retreat, bedankt voor het redigeren van het klinische gedeelte van de tekst.

En dan uiteindelijk deel je ook een coupé, soms eerste klas, soms tweede klas: mijn kamergenoten.

Kamer 1: Angela, Elvira, Wendy, Willem

Kamer 2: Bisheng, Colin, Gwenny, Nadine, Rik, Wouter

Hoe het ook ging met de promotie, het was nooit een straf om naar Rotterdam te komen om met jullie de kamer te delen. In tegendeel. Angela, bedankt voor alle leuke gesprekken. Zelfs nu ik in het WKZ zit laat je weten als je toevallig in de buurt bent. Bisheng, de slapende Chinees. Van monocyten via Chinese politiek tot je belastingformulieren. Onnavolgbaar. Goed je te kennen. De postdocs Colin, Gwenny en Nadine. Van jullie heb ik ontzettend veel geleerd. Colin, het was fijn om je weer tegen te komen in het WKZ, een bekend gezicht in een toch al vriendelijke omgeving. Gwenny, buitengewoon getalenteerd of het nou moleculaire biologie of gitaar betrof, het maakt niet uit. Bedankt voor de lessen. Rik, een beetje Americuh op het eind van mijn promotietraject, zowel in het lab als op de klimmuur. Thanks

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.

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Portfolio

Name PhD student:	Arjan Boltjes
Erasmus MC Department:	Gastroenterology and Hepatology
Research School:	Erasmus Postgraduate school Molecular Medicine
PhD period:	October 2008 – September 2014
Promotor:	Prof.dr. H.L.A. Janssen
Supervisors:	Dr. André Boonstra
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PhD training

General academic and research skills

2008 Course "Stralingshygiëne, deskundigheidsniveau 5B", Erasmus MC zorgacademie, Erasmus MC University Medical Center
2009 Course Molecular Immunology, Erasmus Postgraduate school Molecular Medicine, Erasmus University, Rotterdam
2009 Sensing and signaling by the immune system, Dutch Society for Immunology, Lunteren.
2010 Guiding the action of the immune system: Interactions between the immune system and non-immune tissues, Dutch Society for Immunology, Lunteren.
2011 Course Scientific English Writing, Erasmus Postgraduate school Molecular Medicine, Erasmus University, Rotterdam
2012 Course Virology, Erasmus Postgraduate school Molecular Medicine, Erasmus University, Rotterdam
2012 APCs revisited: the function of antigen presenting cells in health and disease, Dutch Society for Immunology, Lunteren.

Presentations – international conferences

2010 European Macrophage and Dendritic Cell Society (EMDS) Conference, Edinburgh, UK.
Hepatitis B surface antigen activates human Kupffer cells. Poster.
2011 Joint Meeting of the International Cytokine Society (ICS) and the International Society for Interferon and Cytokine Research (ISICR), Florence, Italy.
Peginterferon therapy of chronic viral hepatitis patients differentially regulates plasmacytoid dendritic cell TLR7 and TLR9 function. Poster.
2012 European Association for the Study of the Liver (EASL) Conference: Immune Mediated Liver Injury, Stratford upon Avon, UK.
Hepatitis B virus infection activates Kupffer cells. Poster.

Presentations – domestic conferences

2009 Dutch Society for Immunology (NVVI) Annual meeting, Noordwijkerhout. Ribavirin improves PEG-IFN-induced DC modulation in chronic hepatitis B. Poster.
2010 Dutch Society of Hepatology (NVH) meeting, Veldhoven. Ribavirin improves Pegylated-interferon-α 2a induced dendritic cell modulation in chronic hepatitis B. Poster.
2010 Dutch Society for Immunology (NVVI) Annual meeting, Noordwijkerhout Hepatitis B surface antigen activates Kupffer cells. Poster.
2011 Dutch Society of Hepatology (NVH) meeting, Veldhoven. The interaction between Hepatitis B virus and Kupffer cells leads to immune activation. Poster.
2011 Dutch Society for Gastroenterology (NVGE) and the Dutch Society of Hepatology (NVH): Dutch Liver Retreat, Spier. Hepatitis B virus infection activates Kupffer cells. Oral.
2011 Dutch Society for Immunology (NVVI) Annual meeting, Noordwijkerhout. IFNα therapy of chronic viral hepatitis patients differentially regulates TLR7 and TLR9 activity by plasmacytoid dendritic cells. Poster.
2012 Dutch Society for Immunology (NVVI) Annual meeting, Noordwijkerhout Hepatitis B virus-induced activation of Kupffer cells as first step towards virus-specific immunity? Poster.
2013 Dutch Society of Hepatology (NVH) meeting, Veldhoven. Hepatitis B virus-induced activation of Kupffer cells as first step towards virus-specific immunity? Poster.

Attendance – domestic conferences

2008 Erasmus Liver Day, Erasmus MC University Medical Center Rotterdam, Rotterdam.
2010 Mini-symposium: Virus-Host Interactions and the Regulation of Viral Immunity, Erasmus MC University Medical Center Rotterdam, Rotterdam.

Scientific awards and grants

2010 Travel grant: Dutch Society for Immunology
2011 Travel grant: Erasmus Trustfonds
2012 European Association for the Study of the Liver (EASL) Bursary.
2013 Dutch Society for Hepatology, section Basic Hepatology (2013) DEGH poster prize for best poster + presentation.

Curriculum vitae

Arjan Boltjes, de auteur van dit proefschrift, werd geboren op 20 oktober 1978 te Leeuwarden. Hij groeide op in Marrum, Stiens en kortstondig in Heerenveen. In 1997 behaalde hij zijn middelbare schooldiploma aan het Christelijk Gymnasium Beyers Naudé te Leeuwarden.

Na uitgeloot te zijn voor de studie geneeskunde begon hij de studie biologie, die hij met veel plezier doorliep tot en met het derde jaar. Na bij de vierde maal loten eindelijk ingeloot te zijn ving hij in 2000 alsnog de studie Geneeskunde aan. In 2004 besloot hij dat, in plaats van coschappen te lopen voor geneeskunde, hij de voorkeur gaf aan onderzoekstages om zijn studie biologie af te ronden.

In 2004 deed hij een onderzoeksstage in het tumor immunologie laboratorium van het universitair medisch centrum Groningen onder begeleiding van dr. Wijnand Helfrich. Dit onderzoek betrof de relatie tussen eigenschappen van EBV-specifieke $CD8^+$ T cellen enerzijds en EBV DNA load anderzijds in longtransplantatie patiënten.

In 2005 volgde hierop een onderzoeksstage in het laboratorium van professor Bjarne Bogen onder begeleiding van dr. Alexandre Corthay in het instituut voor immunologie in het Rikshospitalet en aan de Universiteit van Oslo, in Oslo, Noorwegen. Deze onderzoeksstage betrof het onderzoeken van mogelijke mechanismes waarmee idiotype-specifieke T cellen de groei van de multipele myeloma cellijn MOPC-315 kunnen remmen.

In april en mei 2006 ontving hij een doctorandustitel voor zowel medische biologie als geneeskunde.

Na enige uitstapjes in het bancaire (Rabobank) en publieke (Rijkswaterstaat) wezen besloot hij alsnog in Nederland te beginnen aan een promotieonderzoek. Dit promotieonderzoek is beschreven in dit proefschrift en vond plaats in het laboratorium van professor Harry Janssen, tevens promotor, onder begeleiding van dr. Andrea Woltman en dr. André Boonstra. Laatstgenoemden zijn beide copromotor voor deze promotie.

Sinds 1 mei 2013 is hij aangesteld als wetenschappelijk onderzoeker in de onderzoeksgroep van dr. Femke van Wijk en professor Berent Prakken op de afdeling kinderimmunologie van het universitair medisch centrum Utrecht. Hier doet hij onderzoek naar de rol van antigen-presenterende cellen in jeugdreuma.

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Rik A. de Groen, Arjan Boltjes, Jun Hou, Bi-Sheng Liu, Fiona McPhee, Jacques Friborg, Harry L.A. Janssen, and André Boonstra. IFNλ-mediated IL-12 production in macrophages induces IFNγ production in human NK cells. *Submitted*

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