Dendritic Cells in Hepatitis B Virus Infection

Host-pathogen interaction and immune modulation

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ABBREVIATIONS

ALT	alanine transaminase
APC	antigen presenting cell
APC	allophycocyanin
ASGPR	asialoglycoprotein receptor
CD	cluster of differentiation
CHO	Chinese Hamster Ovary
CMV	cytomegalovirus
CpG ODN	C phosphate G oligodeoxynucleotides
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FAM	fluorescein amidite
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBcAg	HBV core antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human Immunodeficiency virus
HLA	human leukocyte antigen
HSV	Herpes Simplex virus
IFN	interferon
IFNAR	interferon-α/β receptor
IFR	interferon regulatory factor
IgG	immunoglobulin G
IL	interleukin
IP-10	10 kDa interferon-gamma-induced protein
ISGF	interferon-stimulated gene factor
Lox	loxoribin
LPS	lipopolysaccharide
LSEC	liver sinusoid endothelial cells
L-SIGN	liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin
mDC	myeloid dendritic cell
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
moDC	monocyte-derived dendritic cell
MR	mannose receptor
mTOR	mammalian target of rapamycin
NK	natural killer
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PEG-IFN	pegylated interferon
PHA	phytohemagglutin
PRR	pattern recognition receptor
RNA	ribonucleic acid
SEM	standard error of the mean
STAT	signal transducers and activators of transcription
TGF	transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
VLP	virus like particle

CHAPTER 1

Introduction

Part of this introduction was adapted from:

Dendritic Cells in Chronic Viral Hepatitis B and C: Victims or Guardian Angels?

Andrea M. Woltman

Andre Boonstra

Harry L.A. Janssen

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History

The discovery of the Hepatitis B virus (HBV) is a typical example of the role of serendipity in scientific progress. In 1967, Dr. Blumberg investigated the link between inherited traits and susceptibility to diseases by taking blood samples from native populations all over the world. These blood samples were tested for the presence of different serum proteins using antibodies derived from blood of haemophiliac patients. Dr. Blumberg reasoned that the immune system of these patients must recognize all blood serum proteins as a result of the blood transfusions they underwent. Using this technique, he discovered a match between an antibody from a haemophiliac patient in New York and a blood serum protein from an Australian aboriginal, which he called Australian antigen. Combination of this finding with research and clinical observations led to the insight that the Australian antigen caused hepatitis or liver inflammation, which in turn led to the discovery of HBV by the virologist Dr. Dane in1970. Two years later, a blood test to identify HBV and a vaccine against the virus were invented. In 1976, Dr. Blumberg was rewarded the Nobel Prize in medicine for this discovery [1].

Hepatitis B virus

HBV is classified as the prototypic family member of the *Hepadnaviridae* and belongs to the genus *Orthohepadnavirus* within this family [2]. All members of this family infect the liver as primary target leading to acute or chronic hepatic infections. The infectious Hepatitis B virion, also called Dane particle, appears in electron microscopy as a 45 nm sphere. HBV contains an outer envelope, which surrounds the nucleocapsid containing the viral DNA. The HBV genome is relatively small. The 3200 bases of the partially double-stranded relaxed-circular DNA encode all viral proteins with four open reading frames: S (surface antigens), C (core antigen and HBeAg), P (polymerase) and X (HBx protein). HBV surface antigens, or HBsAg, consist of small, middle and large surface glycoproteins composed of three domains: preS1, preS2 and S (Fig. 1). The nucleocapsid comprises the viral DNA and the polymerase enzyme surrounded by the structural protein HBV core antigen (HBcAg) [2]. The function of HBeAg and HBx are still under debate. HBeAg is secreted in the circulation, but is dispensable for *in vivo* infection. HBx is required for the establishment of an infection *in vivo*, but is not necessary for viral replication in cell line models. HBV replication starts with the repair of the partially double-stranded genome into covalently closed circular DNA. HBV uses host polymerases to transcribe the viral DNA into several viral RNA intermediates. The smaller mRNAs are translated into viral proteins.

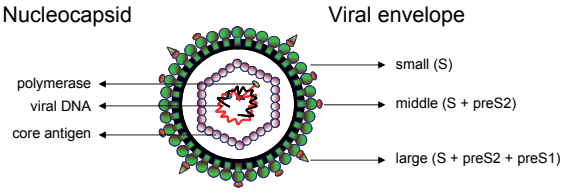

Fig. 1 Schematic representation of HBV.
Indicated is the nucleocapsid containing viral DNA and polymerase, surrounded by the HBsAg envelope consisting of small, middle and large surface antigens.

The largest viral mRNA or pregenomic RNA is packaged together with the viral polymerase into a core particle, where the viral polymerase converts the viral RNA into viral DNA by reverse transcriptase. Finally, these core particles bud through the endoplasmatic reticulum where they acquire the surface envelop and are released as progeny virus particles. Next to HBeAg and infectious virions, HBV infected hepatocytes secrete large amounts of nucleocapsid-free subviral particles, consisting of the small and middle surface antigens. These HBsAg subviral particles form spheres and filaments with a diameter of 22 nm and outnumber the infectious virions more than 100-fold, accumulating up to 100 µg/ml in peripheral blood of HBV infected patients [2].

Viral entry

One of the key questions in HBV biology focuses on the attachment and entry of HBV into the main target cell for infection, the hepatocyte. Several putative binding partners for HBV surface antigens have been described, such as human serum albumin [3], asialoglycoprotein receptor [4], heparin [5] and mannose binding lectin [6], but their exact role in HBV attachment and uptake remains unclear [7]. It is well known that the preS1 domain of the surface antigen is essential for infection of hepatocytes [8,9]. Inhibition of HBV infection can be achieved using preS1 peptides, but inhibition of binding of HBV particles to hepatocytes requires much more stringent conditions [10]. This discrepancy suggests that HBV is first trapped on the hepatocyte by an abundantly expressed low affinity receptor and subsequently enters the cell via a yet undefined high affinity receptor, which can be blocked by preS1 peptides [7]. Since HBV is known to interact with heparin, candidates for the low affinity docking receptor are heparan sulphate proteoglycans, enriched in the liver within the space of Dissé and indispensable for establishment of HBV infection *in vitro* [11].

Course of an HBV infection

HBV can be transmitted by perinatal, percutaneous or sexual exposure. Four to seven weeks post-infection, HBV can be detected in the liver and serum (up to 10^9-10^{10} copies/ml), and has infected most hepatocytes [12,13]. During the acute phase, an efficient anti-viral immune response of the patient will entirely clear the virus. This response involves both the innate and the adaptive immune system, leading to a self-limiting HBV infection. In the absence of a competent immune response, the acute infection can become chronic. The risk of developing chronic HBV infection after acute exposure is determined by age, the route of infection and the dose of inoculum, ranging from 90% in newborns to less than 10% in adults [14,15]. Worldwide, more than 2 billion people have been infected with HBV and in 400 million people this infection became chronic [16]. Although HBV is a non-cytopathic virus, the continuous attacks of infected hepatocytes by cells of the immune system attempting to clear the virus will cause chronic inflammation of the liver. Over the course of approximately 30 years, chronic HBV infection may as a result lead to progressive liver injury with an increased risk of developing liver

cirrhosis, liver failure and liver cancer [15]. Diagnostic liver function tests include histological examination of liver biopsies to determine liver inflammation and fibrosis state and serum measurement of alanine transaminase (ALT), a liver enzyme that is released in the circulation upon liver damage. Approximately 500.000 to 1.2 million people die annually from the consequences of a chronic HBV infection [17].

Treatment of chronic HBV infections

The goal of antiviral therapy of chronic HBV patients is to achieve disease remission by suppressing HBV replication before irreversible liver damage has occurred. The long term treatment goals are to eliminate the HBV infection, prevent progression of liver injury to liver cancer and promote patient survival. Clinical endpoints of antiviral therapy include HBeAg seroconversion (loss of HBeAg with appearance of anti-HBeAg), HBsAg seroconversion, decline in serum HBV DNA below the limit of detection, normalisation of ALT level and improvement of liver histology [18]. Treatment options for chronic HBV can be divided in two categories: immune modulators and HBV replication inhibitors. The first comprises therapy with the cytokine interferon alpha (IFN-α), which has immunomodulatory, anti-proliferative and anti-viral activity [19]. In 2001, a pegylated form of IFN-α (PEG-IFN-α) was developed which has a prolonged half life due to the presence of a polyethylene glycol polymer [20]. PEG-IFN-α treatment leads to sustained response in approximately 20 - 45% of patients, depending on HBV genotype and HBeAg positivity [21,22]. The major advantage of PEG-IFN-α therapy is the relative high rate of sustained response after treatment discontinuation [23,24]. Major disadvantages are the subcutaneous administration and frequent side-effects, mainly flu-like symptoms, cytopenia and psychiatric adverse events [18]. Therefore it would be beneficial if response or non-response to therapy could be predicted at an early stage. Tenofovir, adefovir, entecavir, telbivudine and lamivudine are the current nucleos(t)ide analoques on the market, belonging to the class of HBV replication inhibitors which directly inhibit HBV polymerase [22]. Advantages of this line of therapy are the oral administration, rapid decline in HBV viral load and minimal side effects. The main disadvantages are the risk of development of resistant mutants and the likely need for indefinite therapy, since sustained response is not frequently achieved [18]. Thus, although the currently available treatment strategies are effective in the suppression of HBV replication and subsequent prevention of liver damage, new treatment strategies should be aimed at immunologic control and ultimately complete eradication of the virus. A better understanding of the immunological mechanisms of chronic HBV infection is essential in the development of new treatment strategies.

Dendritic cells in the anti-viral immune response

The first line of defence after infection of the host by a pathogen is formed by the innate immune system, which involves dendritic cells (DC), macrophages, natural killer cells, cytokines and complement components, limiting the spread of the pathogen and initiating an adaptive immune response. In contrast to the immediate and non-specific action of the innate immune

response, the adaptive immune response is specific, long-lasting and protective, mediated by B and T cells residing primarily in the lymph nodes.

Both macrophages and DC continuously sample the environment for potentially dangerous microbes. Upon recognition, the main role of macrophages is to phagocytose and destroy the pathogen, while DC are specialized in communication of the intruder signal to cells of the adaptive immune system. Viruses may activate DC directly through pattern recognition receptors on DC including C-type lectins and Toll-like receptors (TLR) [25,26], leading to internalization of the virus within early endosomes (Fig. 2). DC can also be indirectly activated by viruses through the capture of viral products and by responding to cytokines produced by other cells in response to viral infection [27]. DC integrate these signals and process the captured viral products into viral peptides by fusion of the early endosomes with lysosomes. The virus-derived peptide is then presented to T cells, leading to induction of virus-specific T and B cell responses. The DC is thus a key component in bridging innate and adaptive immunity [28,29]. They not only play a key role in initiating, but also in controlling the magnitude and the quality of adaptive immune responses [28-31].

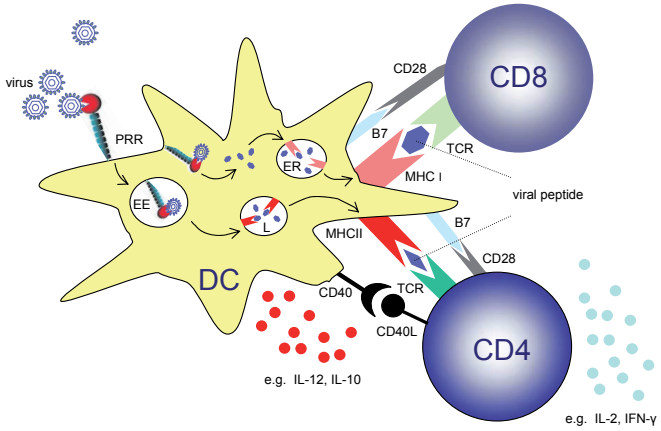

Fig. 2 The role of dendritic cells in pathogen recognition, uptake and presentation to T cells.
Upon recognition of a pathogen by a pathogen recognition receptor on the surface of the dendritic cell, the complex is internalized into early endosomes. After fusion with a lysosome, the pathogen is degraded into peptides which are presented on MCH II molecules to the T cell receptor of CD4 (or: helper) T cells. Pathogen recognition also leads to activation of the dendritic cell, leading to upregulation of co-stimulatory molecules B7 (CD80 or CD86) and CD40. T cell activation only occurs if a co-stimulatory signal is present, coming from either B7-CD28 or CD40-CD40L interaction. In case the pathogen is present in the cytosol of the dendritic cell, the proteasome will degrade it into peptides which are transported to the MHC I molecules in the endoplasmatic reticulum. Peptide-MCH I complexes are transported to the dendritic cell surface, where the peptide is presented to the T cell receptor of CD8 (or: cytotoxic) T cells. CD8 T cell activation only occurs if the co-stimulatory signal and cytokines produced by activated CD4 T cells (IL-2, IFN-γ) are present. Cytokines produced by the dendritic cell can either stimulate (IL-12) or inhibit (IL-10) the immune response. DC=dendritic cell, PRR=pathogen recognition receptor, EE=early endosome, L=lysosome, ER=endoplasmatic reticulum, MHC=major histocompatibility complex, TCR=T cell receptor, IL=interleukine, IFN=interferon.

Induction of IFN-γ-producing T helper1 (Th1) cells, which are crucial in anti-viral immunity, requires recognition of viral peptide presented by major histocompatibility complex II (MHC II) by the T cell receptor on the CD4 T helper cell (signal 1; Fig. 2). Next, a co-stimulatory interaction between B7 molecules on the DC and CD28 on the T cells is required (signal 2). The co-stimulatory molecules CD80 and CD86 belong to the B7 family and are upregulated following DC activation. Co-stimulation may be enhanced through interaction between CD40 and CD40L. Next to signal 1 and 2, cytokines including IL-12 and IFN-α produced by the DC further differentiate the activated CD4 T helper cells into potent effector Th1 cells (signal 3) [32]. In case the pathogen resides in the DC cytosol, it is processed into viral peptides by the proteosome. These viral peptides are then transported into the endoplasmatic reticulum, where they are incorporated within MHC I molecules and finally presented to the T cell receptor on cytotoxic T cells [33]. Cytotoxic T cells need both the co-stimulatory signal and the cytokines produced by the Th1 cells to expand and differentiate into effector cells that can directly kill virus-infected neighbouring cells. DC that are not properly activated and arrested at either an immature or a semi-mature state, lack their co-stimulatory function and production of pro-inflammatory cytokines, and may induce regulatory T cell responses [34]. These regulatory T cells modulate the activity of multiple cells of the immune system by suppressing their effector function [35]. In addition, regulatory cytokines e.g. IL-10 and TGF-β, produced by a wide range of cells strongly suppress DC and macrophage function, which further hampers the induction of an efficient anti-viral response.

Dendritic cell subsets

Human DC can be categorized in two main subsets including myeloid DC (mDC) and plasmacytoid DCs (pDC) [36]. Blood contains both mDC and pDC at frequencies of 0.5-1.0% and 0.2-0.5% of peripheral blood mononuclear cells, respectively. The different DC subsets not only differ in surface markers, but also in their response to pathogens, antigen processing and their capacity to activate T cells [37,38]. mDC induce strong T cell proliferation and initiate anti-viral Th1 responses via the production of IL-12. In contrast to mDC, pDC are poor inducers of T cell proliferation, but are best known for their rapid and high level of type I IFN production in response to TLR7 and TLR9 triggering by single-stranded RNA or DNA containing CpG motifs commonly found in viruses and bacteria [39]. The secretion of excessive amounts of IFN-α, but also other cytokines, has a strong direct anti-viral effect. In addition, type I IFN directly influences immune cells allowing further priming of anti-viral immunity [40].

Dendritic cells in the immunotolerant environment of the liver

The liver has a function in digestion, detoxification and production of plasma proteins. The parenchymal cells, the hepatocytes, are the main contributors to the metabolic function of the liver. Hepatocytes are separated from the sinusoidal lumen by the space of Dissé and an endothelial cell layer. Lymph is collected within the space of Dissé and transported to the celiac lymph nodes. The liver endothelial consists of liver sinusoidal endothelial cells (LSEC).

LSEC are fenestrated, thereby allowing molecules to diffuse from the sinusoidal blood to the hepatocytes. One of the proposed mechanisms by which HBV in the circulation reaches the hepatocytes is through these LSEC fenestrations [41]. Other non-parenchymal antigen presenting cells are the liver macrophages or Kupffer cells, which can penetrate the endothelial layer and the space of Dissé to reach the hepatocytes, and the hepatic DC [42]. These cell types might also be involved in HBV dissemination, similar to HIV transport by DC for trans-infection of T cells [43] and HCV capture by LSEC promoting infection of hepatocytes [44]. Hepatic DC are usually found in the portal triad and the central veins and can migrate through the LSEC into the space of Dissé to travel to the celiac lymph nodes while maintaining immature [45]. Blood coming from the portal vein is so rich in dietary proteins, components of commensal intestinal flora and environmental toxins that the local immune response in the liver is tightly regulated to prevent chronic inflammation of the liver. Several mechanisms may contribute to this tolerant liver environment: antigen presentation by LSEC to CD8 T cells leads to tolerance rather than effector functions, with low IL-2 and IFN-γ production [46]; Kupffer cells and LSEC continuously express the immunosuppressive cytokines IL-10 and TGF-β [47]; and hepatic DC show increased IL-10 production compared to blood-derived DC [48]. As a consequence of this tolerant environment, the liver is a relatively easy target for chronic viral and parasitic infections [49].

Dendritic cells in chronic hepatitis B patients

Several studies, especially those examining patients with advanced fibrosis or cirrhosis [50-53] describe a decrease in circulating blood DC numbers in chronic HBV patients compared to healthy individuals [50-55]. Liver inflammation, as reflected by increased serum ALT levels, was reciprocally correlated with the number of both circulating mDC [52,56] and pDC [57]. This is in line with the observed increased numbers of intrahepatic DC in the portal areas of the liver of chronic HBV patients [53,56]. Since no significant correlation with HBV DNA levels are reported, altered circulating DC numbers are most likely related to altered migration of the cells, which may in turn be related to the phase of disease.

In addition to a decrease in circulating DC numbers, functional deficits in DC or DC precursors from chronic HBV patients may contribute to the impaired HBV-specific immune response as well. Indeed, *ex vivo* examination of mDC of chronic HBV patients showed some phenotypic and, more pronounced, functional alterations compared to mDC of healthy controls [54-56,58]. Upon stimulation, mDC of chronic HBV patients produced less IFN-ß and TNF-α compared to mDC of healthy controls, without significant changes in IL-6, IL-1ß and IL-12 [54]. Functional modulation of DC function may have important consequences for the regulation of T cell responses. Several studies showed indeed that mDC from chronic HBV patients are less efficient in inducing T cell proliferation *in vitro* than mDC isolated from healthy individuals [54,58,59].

Type I IFN production by pDC is characteristic during viral infections [39]. Impaired IFN-α production by pDC from chronic HBV patients has been shown after stimulation with SAC [54],

HSV [51], or DNA containing CpG motifs [53,57,60] as compared to healthy controls. Loss of serum HBeAg during successful treatment of patients with potent anti-viral drugs was suggested to be responsible for partial restoration of circulating pDC numbers and the impaired IFN-α production [51]. Whether PEG-IFN-α therapy will also enhance the impaired pDC function of chronic HBV patients is currently unknown.

The functionality of DC subsets could be affected by the interaction of surface receptors on DC with the virus or its viral proteins, but also as a consequence of infection of the cells or their precursors. Although HBV is generally considered to be highly hepatotropic, small amounts of HBV-specific nucleic acids can be detected in many extra-hepatic tissues during acute and chronic infection [61-64]. Whether human cells other than hepatocytes can support steps of HBV replication is still controversial. Even though DC are likely to be infected in animal models of hepadnavirus infection [65], productive HBV replication appears to be unlikely in chronic HBV patients, as only HBV DNA could be detected but no evidence was found for viral replication in circulating mDC and pDC [54,55], nor in monocyte-derived DC [66]. Thus, although HBV is incapable of replicating in DC, the binding and uptake of viral particles by these cells may be responsible for the impaired function of DC in HBV infected individuals.

AIMS AND OUTLINE OF THIS THESIS

This thesis focuses on the role of DC in chronic HBV infection. Many studies indicate a functional deficit in DC of chronic HBV patients, but it is unclear whether this is caused by a direct interaction between the DC and the virus or viral proteins or by a more indirect effect of the chronic inflammation state of the liver. The DC-HBV interaction and the subsequent immunomodulatory effect on the anti-viral immune response are investigated and discussed here. In addition to reported deficiencies in CD4 T cell stimulation, **chapter 2** presents data on the reduced capacity to expand HBV-specific CD8 T cells displayed by mDC of chronic HBV patients. This reduced capacity to expand CD8 T cells could be abolished by neutralizing IL-10, suggesting that IL-10 is an important player in HBV induced viral persistence and disease chronicity. **Chapter 3** describes the direct tolerogenic effect of HBV and HBsAg on mDC function: the T cell stimulatory capacity was reduced, as assessed by T cell proliferation and IFN-γ production. The receptors involved in the recognition of HBV and HBsAg by mDC were also investigated. Interaction between DC and viruses through the pathogen recognition receptor DC-SIGN has attracted much attention lately. Although HBV meets the criteria for DC-SIGN binding, DC-SIGN is excluded as a HBV receptor in **chapter 4**. In **chapter 5** a role for the mannose receptor in the uptake of HBsAg by mDC is presented. Analysis of blood- and liver-derived DC of chronic HBV patients shows that interaction with HBsAg predominantly occurs at the main site of infection, the liver. **Chapter 6** describes the inhibitory effect of HBV on pDC function. TLR9 triggering is directly inhibited by HBV, leading to reduced pDC activation and diminished levels of IFN-α and other pro-inflammatory cytokines.

The inhibitory effects on TLR9-mediated pDC function and activation are further enhanced in presence of monocytes. **Chapter 7** presents the results of a longitudinal study on the effect of PEG-IFN-α-2a therapy on pDC function of chronic HBV patients. The response to TLR7 and TLR9 specific ligands is differentially regulated during PEG-IFN-α-2a therapy by a direct effect on TLR7 and TLR9 expression levels. This effect is directly caused by PEG-IFN-α therapy and could not be attributed to change in HBV viral load levels. Together, this thesis provides evidence for the direct interference of HBV with both mDC and pDC as part of the immune escape mechanism leading to maintenance of a chronic HBV infection.

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CHAPTER 2

Reduced expansion of HBV-specific CD8 T cells by dendritic cells from chronic Hepatitis B patients: a role for IL-10?

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ABSTRACT

Chronic hepatitis B virus (HBV) infection is characterized by a relatively weak, antigen restricted or even undetectable T cell response to the virus. Dendritic cells (DC) play an important role in the induction of T cell responses. Previously, it was shown that mDC of chronic HBV patients are impaired in their maturation and allostimulatory capacity. It is unclear whether these hampered mDC can affect the expansion of HBV-specific CD8 T cells. We therefore analysed the capacity of mDC from chronic HBV patients to expand HBV-specific CD8 T cells. The expansion of HBV-specific CD8 T cells from a resolved HBV patient was significantly reduced by mDC of chronic HBV patients compared to mDC of healthy controls, both in whole PBMC cultures and in purified CD8 T cell cultures. Interestingly, purified mDC of chronic HBV patients produced more IL-10 compared to mDC of healthy controls. Neutralization of IL-10 completely restored the reduced expansion of HBV-specific CD8 T cells. In contrast to the reduced capacity of HBV-specific CD8 expansion by HBV patient-derived mDC, the expansion of CMV-specific CD8 T cells was only slightly inhibited compared to healthy control-derived mDC. In conclusion, these preliminary results indicate that mDC of chronic HBV patients display a preferential reduced capacity to expand HBV-specific CD8 T cells, which was IL-10 dependent.

INTRODUCTION

Chronic infection with hepatitis B virus (HBV) leads to an inflammatory liver disease of variable severity and is world-wide a major cause of liver failure and hepatocellular cancer [1,2]. The immunopathogenesis of chronic HBV infection is unclear up till now and considered to be the result of a complex interaction between a replicating non-cytopathic virus and a down-regulated antiviral immune response. To recover from an HBV infection, both a strong humoral and cellular immune response is required. During an acute infection the class I- and class II-restricted T cell responses to the virus are vigorous, polyclonal and multispecific [3-5], while such responses are relatively weak, antigen restricted or even undetectable in chronically infected patients [6,7].

Dendritic cells (DC) are specialized cells critical for the development of immunity to HBV. They are capable of directing the magnitude, polarity and effector function of the T cell response [8]. DC possess the capacity to capture and process antigens and present these in the context of co-stimulatory and HLA class II molecules to T cells. Depending on their maturation status, represented by the expression level of co-stimulatory and HLA molecules and the capacity to produce pro-inflammatory or anti-inflammatory cytokines, DC can induce either immunity or tolerance [9,10]. Immature and semi-mature DC are associated with tolerogenic responses. In the context of HBV a defect in the maturation process of DC may lead to tolerogenic T cell responses and HBV persistence. We and others have previously shown that a specific subset of DC, the myeloid dendritic cells (mDC), of chronic HBV patients are indeed impaired in their capacity to mature compared to mDC of healthy controls, as shown by a decreased capacity to upregulate co-stimulatory molecules, produce pro-inflammatory cytokines and stimulate allogeneic T cells [11,12]. Reducing the viral load by the nucleotide analogue adefovir dipivoxil resulted in an improvement of numbers and functionality of mDC. More specifically, virus reduction resulted in increased allostimulatory capacity and the capacity of mDC to produce IL-12 and TNF-α, while the capacity of mDC to produce IL-10 was decreased [13]. In a later study we have shown that the presence of both HBV particles and HBV proteins has a direct effect on mDC function [14]. Taken together this indicates an important role for the presence of viral particles and/or viral proteins in dampening the immune response with viral persistence as a result.

Up till now, it is unknown what the contribution is of dysfunctional mDC from chronic HBV patients to the presence and expansion of HBV-specific CD8 T cells. CD8 T cells are extremely important for clearance of HBV infected hepatocytes, but in chronic HBV patients they are usually very difficult to find [15-17]. In this study we have therefore developed an experimental setup to determine the capacity of mDC of chronic HBV patients to expand HBV-specific CD8 T cells.

MATERIALS AND METHODS

Patients

Peripheral heparinized blood samples were obtained from 14 HLA-A2$^+$ patients with chronic hepatitis B (Table 1). All patients were negative for antibodies against human immune deficiency virus, hepatitis C and hepatitis D. None of the patients were treated for chronic HBV infection or received any other medication 6 months previous to blood sampling. All patients had biopsy proven active chronic hepatitis. The median serum HBV DNA load was $2.0x10^6$ copies/mL (range $1.0x10^3$ – $4.9x10^9$) and median alanine transaminase level (ALT) was 28 U/L (range 15 – 262). Seven patients were HBV envelope antigen (HBeAg) positive and 7 patients were HBeAg negative and had antibodies to HBeAg. A control group, matched for age and gender, consisted of 14 healthy subjects who had no evidence of exposure to HBV (HBV surface antigen (HBsAg) negative). In addition, peripheral blood was obtained from a HLA-A2$^+$ person that had resolved an acute HBV infection and has antibodies to HBsAg and a HLA-A2$^+$ person with positive serology for cytomegalovirus (CMV). The study was approved by the local ethics committee and all patients and controls in the study gave informed consent before blood donation.

Table 1 Characteristics of chronic HBV patients and healthy controls

	Chronic HBV patients	Healthy controls
Sex (m/f)	14 (10/4)	14 (9/5)
Age (years)*	36 (21-49)	28 (24-53)
HBV DNA*	$2.0x10^6$ ($1.0x10^3$-$4.9x10^9$)	-
ALT*	28 (15-262)	-
HBeAg$^+$/anti-HBeAg$^+$	7/7	-

* median (range)

Virological assessments

Serum HBsAg, HBeAg, and anti-HBe were determined quantitatively using the IMX system (Abbot Laboratories, North Chicago, IL, USA) according to the manufacturers' instructions. Serum HBV DNA was determined using a HBV monitor assay (Roche Applied Science, Penzberg, Germany; detection limit $1x10^3$ geq/mL). When the serum HBV DNA was below $1x10^3$ geq/mL HBV DNA the assay was repeated using an in-house developed TaqMan PCR (detection limit 373 geq/mL) [18].

HLA typing

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by density gradient centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Screening for HLA-A2 haplotype positivity was performed by staining PBMC with an anti-HLA-A2 antibody derived from a hybridoma (clone BB7.2, ATCC HB-82). Staining was fol-

lowed by an FITC-conjugated rabbit anti-mouse anti-IgG (Dako, Glostrup, Denmark) secondary antibody and flow cytometric analysis (FACScalibur, Becton Dickinson, San Jose, CA, USA).

Isolation of myeloid dendritic cells and CD8 T cells

MDC were isolated from fresh PBMC of HLA-$A2^+$ chronic HBV patients and healthy controls by positive immunomagnetic selection using the mini-MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturers' instructions as previously described [11]. The cells were resuspended in culture medium consisting of RPMI 1640 (Bio Whittaker, Verviers, Belgium) containing 10% fetal calf serum (Hyclone, Logan, UT) and penicillin/streptomycin (Gibco, Paisley, UK). PBMC and purified CD8 T cells were isolated from a HLA-$A2^+$ healthy individual who resolved an acute HBV infection and a HLA-$A2^+$ CMV-positive healthy individual. CD8 T cells were isolated from fresh PBMC by negative selection using the CD8 T cell isolation kit II (Miltenyi Biotec). Isolation was performed according to the manufacturers' instructions. After isolation the cells were immediately frozen in medium containing 10% DMSO and stored at –135°C until further use. Purity of $BDCA1^+CD19^-$ mDC and CD8 T cells was determined by flow cytometry and the purity of the cells was more than 90%.

Coculture of myeloid dendritic cells and CD8 T cells

Freshly isolated mDC were cultured in a concentration of $1x10^4$ in 200 mL culture medium and pulsed with HBV core peptide (HBc_{18-27} (FLPSDFFPSV); ProImmune, Oxford, UK) or a CMV peptide ($CMV_{510-522}$ pp65; kindly provided by Dr. van Baarle) in a flat bottom 96-wells plate. The HBc_{18-27} peptide was used at 4 mM and the $CMV_{510-522}$ pp65 peptide at 10 mg/mL. After 2 hours the cells were washed and maturated for 24 hours in culture medium with 25 ng/mL TNF-α (Strahtmann Biotech, Hannover, Germany), 50 ng/mL IL-1ß (Strathmann Biotech) and 500 U/mL GM-CSF (Leucomax, Novartis Pharma, Arnhem, The Netherlands). Cells were washed and PBMC (1.5×10^5 cells in 200 µL) or purified CD8 T cells (0.5×10^5 cells in 200 µL) from the HLA-$A2^+$ resolved HBV or the HLA-$A2^+$ CMV-positive individual were added. An IL-10 neutralizing antibody (BD Pharmingen) or an IgG1 isotype control (BD Pharmingen) was added to the culture in a concentration of 10 mg/mL when indicated. Recombinant IL-2 (20 U/mL) was added at the start of each culture and every 3 days of the culture. After a culture period of 9 days, the cells were harvested and the percentage of specific CD8 T cells was measured by flow cytometry. The cells were stained for 30 minutes at 37°C with a HLA-A2 restricted HBc_{18-27} specific tetramer (ProImmune) or a HLA-A2 restricted CMV-pp65 tetramer (provided by Dr. van Baarle), both labeled with a PE conjugate. As a control for staining an APC-labeled HLA-A2 restricted $HBpol_{816-824}$ (SLYADSPSV) specific tetramer was used. The cells were washed and stained for CD3-FITC (Immunotech) or CD3-PE (Becton Dickinson) and CD8-APC (Becton Dickinson) or CD8-FITC (Dako), washed again and analyzed by flow cytometry.

IL-10 ELISPOT

The production of IL-10 by mDC was measured using an ELISPOT kit (Sanquin, Amsterdam, The Netherlands) according to the manufacturers' instructions. Freshly isolated mDC of 5 chronic HBV-patients and 5 healthy controls were cultured in triplicate in a flat bottom plate in a concentration of $4x10^4$ cells in 200 mL of culture medium with 25 ng/mL TNF-α, 50 ng/mL IL-1ß and 500 U/mL GM-CSF or 500 U/mL GM-CSF alone (negative control) for 24 hours at 37ºC. The next day cells were transferred to a flat bottom anti-IL-10-coated 96-wells plate (Millipore, Molsheim, France) and incubated for another 24 hours at 37ºC. As a positive control total PBMC ($2x10^5$ cells per well) of chronic HBV patients or healthy controls were stimulated with 5 mg/mL phytohemagglutin (PHA; Murex, Paris, France). Cytokine spots were visualized by a biotin-labeled antibody against IL-10 and their numbers were established by using a Bioreader 3000 from BioSys (Karben, Germany).

Statistical analysis

Data are expressed as mean±SEM, unless indicated otherwise. Data were analyzed and compared to baseline results with SPSS 11.5 for Windows (SPSS, Chicago, IL) using a Wilcoxon matched pairs signed rank sum test. The Mann-Whitney was used to compare variables between two independent groups of patients. In all analyses a p-value of <0.05 was considered statistically significant.

RESULTS

Reduced expansion of HBcore-specific CD8 T cells by mDC of chronic HBV patients

To investigate the capacity of mDC of chronic HBV patients to expand HBV-specific CD8 T cells, purified mDC of chronic HBV patients or healthy controls were pulsed with HBcore peptide and cultured with total PMBC or purified CD8 T cells from a resolved HBV individual, known to have HBV-specific memory T cell responses. Expansion of HBV-specific CD8 T cells was determined by flow cytometry using an HBcore-specific pentamer. Figure 1 shows flow cytometry dot plots of a representative experiment. Pulsing mDC with HBcore-specific peptide showed clear expansion of HBcore-specific CD8 T cells. No aspecific binding was observed when a pentamer recognizing HBV polymerase-specific CD8 T cells was used (Fig. 1).

The capacity of purified mDC, isolated from chronic HBV patients, to expand HBcore-specific CD8 T cells was first studied in PBMC cultures. Figure 2 shows that the expansion of HBcore-specific CD8 T cells is significantly less by mDC isolated from chronic HBV patients as compared to mDC from healthy controls. The percentage of HBcore-specific CD8 T cells was more than two-fold reduced after stimulation with mDC derived from chronic HBV patients compared to healthy controls (respectively 4.08 ± 0.80 versus 8,57 ± 1.26 (mean ± SEM)). To exclude possible influence of CD4 T cells or other cells on the expansion of HBV-specific CD8 T cells, purified CD8

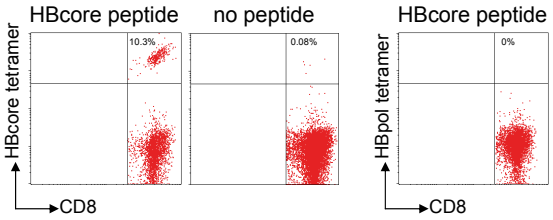

Fig. 1 Flowcytometry dot plots of co-cultures of mDC and CD8 T cells.
Left panel: When mDC were pulsed with HBcore-specific peptide an expansion of HBcore-specific CD8 T cells was detected using a double staining for CD8 and a labeled HLA-A2 restricted HBcore18-27 specific pentamer. Middle panel: Expansion of HBcore-specific CD8 T cells with a HBcore18-27 specific pentamer was not detectable when mDC were pulsed without peptide. Right panel: Staining of HBcore-specific CD8 T cells with a labeled HLA-A2 restricted HBpol18-27 specific pentamer showed no aspecific binding.

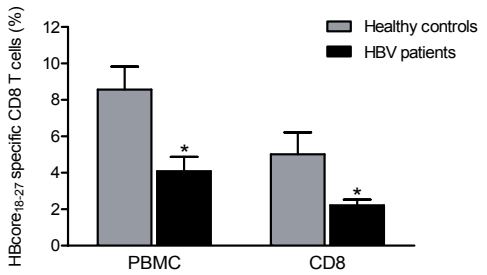

Fig. 2 The capacity of mDC isolated from chronic HBV patients and healthy controls to expand HBc-specific CD8 T cells from a resolved HBV patient.
Purified mDC were pulsed with HBcore18-27 peptide for 2 hours, washed and cultured with total PMBC or purified CD8 T cells from a HLA-A2 matched resolved HBV patient. Gray bars represent mDC isolated from healthy controls (n=14) and black bars are mDC from chronic HBV patients (n=14). HBcore18-27 CD8 T cells are given as % of total CD8 T cells. Data are expressed as mean±SEM, *p<0.05.

T cell cultures were also used. The results using purified CD8 T cells showed a similar reduced expansion of HBcore-specific CD8 T cells by mDC of chronic HBV patients compared to mDC of healthy controls (respectively 2.22 ± 0.31 versus 5.02 ± 1.19 (mean ± SEM) Fig. 2).

Myeloid dendritic cells of chronic HBV patients produce IL-10

IL-10 plays an important role in inducing viral persistence [19-21]. To investigate whether IL-10 could play a role in the reduced expansion of HBV-specific T cells, we determined the production of IL-10 in the supernatant of mDC from chronic HBV patients and healthy controls during 24 and 48 hours of culture with maturation factors. Unfortunately, the concentration was below the detection limit of the kit (data not shown). Therefore, a more sensitive technique was used. Purified mDC were cultured with maturation factors for 24 hours after which they were transferred to an IL-10 ELISPOT plate for another 24 hours. An increased number of IL-10 specific spots were found in cultures of mDC of chronic HBV patients as compared to healthy control mDC. It must be noted however that only two out of five patients and healthy controls showed measurable IL-10 spots and that inter-person variability was high (Fig. 3). MDC cultured without maturation factors hardly produced IL-10. As a control for the IL-10 ELISPOT assay, total PBMC

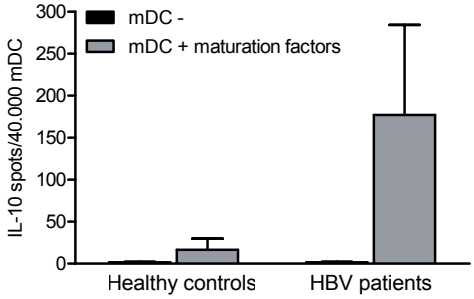

Fig. 3 Production of Il-10 by purified mDC of healthy controls and chronic HBV patients.
Purified mDC (40.000 per well) were cultured with or without maturation factors for 24 hours after which they were transferred to an IL-10 ELISPOT plate for another 24 hours. The number of IL-10 spots per 40.000 cells is depicted. Data are expressed as mean±sd; healthy controls n=2; HBV patients n=2.

of the HBV patients and controls were stimulated with PHA. No difference in IL-10 spots in total PHA-stimulated PBMC was observed between chronic HBV patients and healthy controls (data not shown).

Neutralizing IL-10 restores the reduced expansion of HBcore-specific CD8 T cells by mDC of chronic HBV patients

Next, we investigated whether blocking IL-10 could overcome the reduced CD8 expansion by mDC. For this purpose, during co-culture of mDC and CD8 T cells a neutralizing antibody to IL-10 or isotype matched control antibody was added. As expected seen the minimal IL-10 production, neutralizing IL-10 in the co-culture of mDC of healthy controls had no effect on CD8 T cell expansion. Figure 4 shows that neutralizing IL-10 completely restored the reduced capacity of mDC of chronic HBV patients to expand HBcore-specific CD8 T cells compared to isotype matched and healthy controls.

The expansion of CMV-specific CD8 T cells by mDC of chronic HBV patients is only slightly reduced

In order to examine if the reduced expansion of CD8 T cells by mDC from chronic HBV patients was HBV-specific, the capacity of mDC to expand CD8 T cells specific for CMV was investigated. To this end purified mDC from chronic HBV patients or healthy controls were pulsed with an HLA-A2-restricted CMV peptide pp65 and the expansion of CMV-specific CD8 T cells from a healthy CMV-carrier was determined with a CMV-specific tetramer. The results show that mDC of chronic HBV patients have a slightly reduced capacity to expand CMV-specific CD8 T cells as compared to mDC of healthy controls, in the absence of neutralizing IL-10 or isotype control antibodies (respectively 39.6 ± 3.84 versus 48.2 ± 4.89 (mean ± SEM) Fig. 5). However, no difference in CMV-specific CD8 T cells expansion was observed in the presence of neutralizing IL-10 or isotype control antibodies.

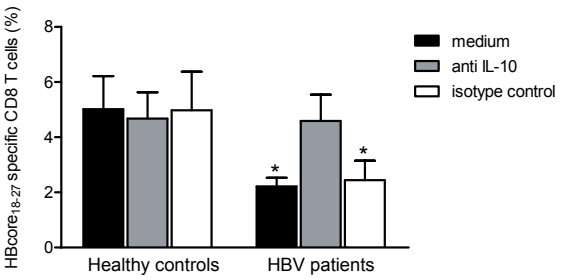

Fig. 4 Effect of IL-10 on HBV-specific CD8 expansion.
Purified mDC of chronic HBV patients (n=14) or healthy controls (n=14) are cultured with HBV-specific CD8 T cells from a resolved HBV patient with addition of neutralizing antibody to IL-10 (gray bars) or an isotype-matched control (striped bars) or culture medium only (black bars). Data are expressed as mean±SEM, *p<0.05.

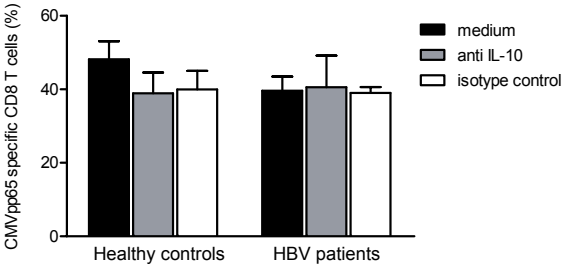

Fig. 5 The capacity of mDC of chronic HBV patients and healthy controls to expand CMV-specific CD8 T cells.
Purified mDC were pulsed with CMV510-522 pp65 peptide for 2 hours, washed and cultured with purified CD8 T cells from a HLA-A2 matched control seropositive for CMV. Data are expressed as mean±SEM; healthy controls n=7; HBV patients n=7.

DISCUSSION

The exact mechanism by which HBV escapes immunity in chronically infected patients is still not known. Previously, we demonstrated significant functional impairment of mDC of patients with chronic HBV infection as compared to healthy volunteers and patients with chronic inflammatory liver disease of non-viral origin [11]. MDC of chronic HBV patients showed a decreased capacity to upregulate co-stimulatory molecules, produce pro-inflammatory cytokines and stimulate allogeneic T cells. Thus far, it is unknown what the consequence of dysfunctional mDC is on the expansion and number of HBV-specific CD8 T cells in chronic HBV patients.

This study shows that mDC of chronic HBV patients are less capable to expand HBcore-specific CD8 T cells as compared to mDC isolated from healthy controls. The reduced expansion was observed using PBMC, as well as using purified CD8 T cells, excluding a possible difference in allostimulatory effect by class II mismatch. Although the difference in the expansion of CD8 T cells was similar using whole PBMC cultures or purified CD8 T cells, the percentages of HBcore-specific CD8 T cells were smaller using purified CD8 T cells as compared to whole PBMC cultures. Most likely this is caused by the production of cytokines such as IL-2 by other cells than CD8 T cells, present in the whole PBMC cultures. In our experiments alloreactivity by class I mismatch was minimal, since there was no significant difference in expansion of HBV-specific CD8 T cells between HBV patients and controls without the addition of peptide i.e. background

response. The use of effector T cells of only one person with a resolved HBV infection is one of the limitations of the present study. Furthermore, testing of a broader panel of HBV peptides could be included as a next step, to observe the expansion of HBV-specific CD8 T cells with other specificity then $HBcore_{18-27}$.

Recent attempts to elucidate the causes of impairment of antiviral immunity have pointed to an important role for the immunomodulatory cytokine IL-10 in the ability to establish viral persistence [19-21]. Induction of IL-10 production by the host during chronic infection appears to be one of the viral means to suppress antiviral immune responses. Especially in the case of a viral infection in which continuous activation of CD8 T cells could lead to extensive, even fatal, tissue damage, dampening of the immune response is very important to improve the outcome for the host. Also in the pathogenesis of chronic HBV a role for IL-10 has been shown [22,23]. These studies showed an increase in IL-10 production in chronic HBV patients non-responsive to IFN-α or IFN-α and ribavirin combination therapy. Various cell types including lymphocytes, macrophages and DC can be involved in IL-10 production. We have previously shown that IL-10 production by mDC was decreased after reduction of the viral load by adefovir treatment, resulting in an increased T cell stimulatory function [13]. The current study shows that an increased number of mDC of chronic HBV patients produced IL-10 compared to mDC of healthy controls as evaluated by ELISPOT assay, although more patients and controls should be included before solid conclusions can be drawn. Neutralization of IL-10 completely restored the impaired induction of HBV-specific CD8 T cells by mDC of chronic HBV patients. Further studies should focus on the phenotype and function of the HBV-specific CD8 T cells with regards to IL10. The sensitivity to IL-10 might be increased by increased expression of the IL-10 receptor for example. Also here it would be important to include HBV-specific CD8 T cells from several patients and investigate CD8 T cells specific for different HBV epitopes.

The reduced expansion was partly HBV specific, since the expansion of CMV-specific CD8 T cells was not significantly reduced and adding neutralizing IL-10 antibody had no effect on the CMV-specific CD8 expansion. The preferential reduced capacity of mDC from chronic HBV patients to expand HBcore-specific CD8 T cells might be explained by the fact that the expansion of CMV-specific CD8 T cells is about 5 to 10-fold more fiercely. The amount of IL-10 produced by mDC of chronic HBV patients is possibly less capable to reduce this major response. Another factor that could possibly play a role is the difference in binding affinity of the two peptides for the A2-restricted T-cell receptor. Ideally, the effect of IL-10 on HBV- and CMV-specific CD8 T cell expansion should be studied using CD8 T cells from the same individual, but in practice it is difficult to find an individual with both an acute resolved HBV and CMV infection.

The pathogenesis behind the functional impairment of mDC is not clear up till now. Reports are available that HBV can be detected on or within mDCs but viral replication in mDC has never been shown [24,25]. On the other hand, recently it was shown viral load reduction by adefovir treatment, lead to a substantial recovery of mDC, as shown by increased production of IL-12 and TNF-α, decreased Il-10 production and an increased allostimulatory capacity. *In vitro*, the

presence of both HBV particles and HBV surface proteins directly influenced the function of mDC isolated from healthy controls [14]. Together this implicates that viral particles or antigens can indeed directly affect the function of mDC. To further support this conclusion, the correlation with HBV viral load and the mDC capacity to produce IL-10 and expand HBV-specific T cells could be calculated for each individual patient included in the extended study.

In vivo the effect of mDC impairment is combined with several other immune modulating effects, such as a defect in CD4 T cell help and the presence of regulatory T cells and therefore the overall end-result is much more dramatic [26,27]. For an efficient CD8 T cell response *in vivo* also an efficient helper T cell response is necessary. Up till now, assessing the capability of mDC to induce a HBV-specific CD4 T cell response has not been done. If mDC and CD4 T cells from the same patients would be used, the defect can be present in mDC as well as in CD4 T cells.

In conclusion, the results presented in this study show that mDC of chronic HBV patients are besides earlier described functional defects also impaired in their capacity to expand preferentially HBV-specific CD8 T cells responses, which was IL-10 dependent. In this case the expansion of preexisting HBV-specific CD8 T cells was studied, but one could imagine that priming of naive HBV-specific responses might be even more affected. Priming of naive responses requires more steps such as uptake, processing and presenting of HBV antigens. *In vivo* the impairment of mDC may lead to a handicapped immune system that is incapable of mounting an efficient immune response against HBV. Novel therapeutic strategies directed against the functional impairment of mDC may be able to overcome HBV chronicity.

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CHAPTER 3

Hepatitis B virus surface antigen impairs myeloid dendritic cell function: A possible immune escape mechanism of HBV

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ABSTRACT

Chronic Hepatitis B virus (HBV) infection is the result of an inadequate immune response towards the virus. Myeloid dendritic cells (mDC) of chronic HBV patients are impaired in their maturation and function, resulting in more tolerogenic rather than immunogenic responses, which may contribute to viral persistence. The mechanism responsible for altered mDC function remains unclear. HBV infected patients display large amounts of HBV particles and viral proteins, especially the surface antigen HBsAg, in circulation, which allows multiple interactions between the virus, its viral proteins and DC. To assess whether HBV directly influences mDC function, the effects of HBV and HBsAg on human mDC maturation and function were investigated *in vitro*. As already described for internalization of HBV by DC, the present study shows that peripheral blood-derived mDC of healthy controls also actively take up HBsAg in a time-dependent manner. Cytokine-induced maturation in the presence of HBV or HBsAg resulted in a significantly more tolerogenic mDC phenotype as demonstrated by a diminished upregulation of co-stimulatory molecules and a decreased T cell stimulatory capacity, as assessed by T cell proliferation and IFN-γ production. In addition, the presence of HBV significantly reduced IL-12 production by mDC. These results show that both HBV particles and purified HBsAg have an immune modulatory capacity and may directly contribute to the dysfunction of mDC in chronic HBV patients. The direct immune regulatory effect of HBV and circulating HBsAg particles on the function of DC can be considered as part of the mechanism by which HBV escapes immunity.

INTRODUCTION

Hepatitis B virus (HBV) infects the liver as primary target, resulting in the majority of cases in self-limiting acute hepatitis, which confers protective HBV specific T and B cell responses [1,2]. Nevertheless, more than 350 million people are chronically infected with HBV, as the result of a complex interaction between the replicating virus and an inadequate immune response. The immune system evolved to recognize and eliminate foreign antigens. However, if the immune system by any reason has become tolerant to HBV, the HBV-specific T and B cell responses are generally undetectable [3,4]. The exact mechanism by which HBV escapes immunity is still not known.

Dendritic cells (DC) play an important role in anti-viral immunity and have the unique capacity to activate naive T cells and stimulate B and natural killer cells [5,6]. Both circulating and tissue-resident immature DC sample the environment for the presence of foreign antigens and upon activation, DC migrate to lymphoid tissues to initiate immune responses. Depending on their maturation status, represented by the expression level of co-stimulatory and HLA molecules and the capacity to produce pro-inflammatory cytokines, DC can induce either immunity or tolerance [7,8]. Immature and semi-mature DC are associated with tolerogenic responses, so in the context of HBV a defect in the maturation process of DC may lead to tolerogenic T cell responses and HBV persistence. We and others have previously shown that a specific subset of DC, the myeloid dendritic cells (mDC), of chronic HBV patients are indeed impaired in their capacity to mature compared to mDC of healthy controls, as shown by a decreased capacity to upregulate co-stimulatory molecules, produce pro-inflammatory cytokines and stimulate T cells [9,10]. Monocyte-derived DC (moDC) are also reported to be functionally impaired by the presence of HBV [11,12] although deficits remained minor in other studies [13]. Whether HBV directly interferes with DC function is not known. In theory, the different steps of binding, uptake and subsequent replication of HBV could all compromise DC function. Contradictory results have been reported about the possible presence and active replication of HBV in *in vitro*-generated moDC of chronic HBV patients [11,14]. Concerning circulating mDC, viral DNA could be detected in a subset of chronic HBV patients but no evidence was found for viral replication in these cells [15]. These observations have been confirmed and expanded by a study of Untergasser *et al*. that demonstrates that moDC are capable of internalizing HBV, but do not allow HBV replication [16].

Although HBV seems to be incapable to replicate in DC, the binding and uptake of viral particles by these cells may introduce some functional changes. The liver and peripheral blood of HBV infected individuals can reach levels of 10^9-10^{10} infectious particles per ml, which allows multiple interactions between the virus and DC. In addition, the liver and peripheral blood of HBV patients contain large amounts of viral proteins, especially the HBV surface antigens [17,18]. The HBV envelop consists of small (S), middle (M) and large (L) surface antigens, generally referred to as HBsAg. All three surface antigens contain a common S domain, both M and

L proteins contain a preS2 domain and L exclusively contains a preS1 domain. S is the main component of both HBV virions and HBsAg subviral particles, while M and L are highly enriched on HBV virions. HBsAg is secreted from infected hepatocytes as subviral particles, which can accumulate up to 100 µg/ml in peripheral blood. The high amounts of HBsAg in the circulation of patients can theoretically contribute to the hampered immune response. Whether HBsAg interacts with DC is not known. Therefore, the aim of the present study is to assess the nature of interaction between HBsAg or HBV and myeloid DC, and the possible role of this interaction for the persistence of HBV infection.

MATERIALS AND METHODS

HBV surface antigens

The following viral antigens were kindly provided by M. van Roosmalen (bioMérieux, Boxtel, The Netherlands) and listed in Fig. 1: pepsin treated HBsAg particles immunopurified from sera of patients infected with HBV of serological subtypes *ad* and *ay* (patient serum derived HBsAg; aa 1-226) [19] and recombinant (r)HBsAg (aa 1-281, subtypes *ad* and *ay*) purified from transfected Chinese Hamster Ovary (CHO) cells. Cell lysates of non-transfected CHO cells served as a negative control. Representative Coomassie stained protein gels and western blots of patient serum derived and recombinant HBsAg, provided by M. van Roosmalen, show the native p24 and the glycosylated gp27 forms of the S protein and the two glycosylated forms of the M protein, the latter only present in recombinant HBsAg. The higher molecular weight bands represent dimer and trimer forms of HBsAg (Fig. 1B). HBsAg was used at a final concentration of 1 µg/ml.

HepG2.2.15 derived HBV

HepG2.2.15 cells [20,21] were grown until confluence in Williams' E medium (Gibco, Paisley, UK) supplemented with 5% fetal calf serum (FCS, Hyclone, Logan, UT) and HBV particles were concentrated from the medium as described before [16]. As a negative control, the same procedure was followed with supernatant from untransfected HepG2 cells. Unless otherwise stated, HBV particles were used at a multiplicity of infection (MOI) of 100.

Isolation of mDC from peripheral blood

Peripheral blood mononuclear cells (PBMC) were isolated from healthy control blood using Ficoll-Isopaque gradient centrifugation. In total, blood from 24 different healthy donors was obtained (15 male and 9 female; median age (range) 30 (23-54) years). All healthy controls gave written informed consent before blood donation and the institutional ethical committee gave declaration of no objection for this study. Myeloid DC ($BDCA1^+$) were isolated from PBMC by negative depletion with anti-CD19-conjugated microbeads, followed by positive selection using anti-BDCA1-PE and PE-conjugated microbeads using the mini-MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany)

A

	Subtype	Source	Control
HBsAg	ad	Patient serum	-
		CHO	CHO
HBsAg	ay	Patient serum	-
		CHO	CHO
HBV particles	ayw	HepG2.2.15	HepG2

B

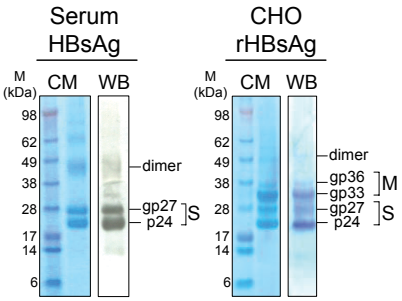

Fig. 1 Characteristics of HBsAg and HBV. (A) Source and subtypes of HBsAg and HBV and available controls. (B) Representative Coomassie stained protein gel (CM) and Western blot (WB) of HBsAg purified from pooled patient serum and recombinant CHO-derived HBsAg.

as described previously [9]. The purity and viability of the isolated mDC were determined by flow cytometry by staining for BDCA1 and CD20 (to ensure the absence of contaminating $BDCA1^{+}CD20^{+}$ B lymphocytes) and trypan blue and 7AAD staining (Becton, Dickinson and Company (BD), Franklin Lakes, USA), respectively. Isolated mDC were cultured in DC medium consisting of RPMI 1640 (Bio Whittaker, Verviers, Belgium) containing 10% FCS (Hyclone, Logan, UT), penicillin (50 IU/ml), streptomycin (50 µg/ml) and GM-CSF (500 U/ml; Leucomax, Novartis Pharma, Arnhem, The Netherlands).

HBsAg binding and uptake by mDC

Isolated mDC were incubated for 2 or 18 hours with rHBsAg in the presence of GM-CSF (500 U/ml, Leucomax) and antigen uptake was stopped by cold wash in PBS containing 1% FCS and 0.02% NaN_3. Intracellular staining was performed according to the manufacturer's protocol (IntraPrep, Beckman Coulter, Fullerton, CA), HBsAg was stained with anti-HBsAg-FITC (recognizing subtypes *ad* and *ay*, Acris Antibodies GmbH, Hiddenhausen, Germany) and detected by flow cytometry (FACScalibur, BD). Negative controls were incubated without antigen or with antigen at 4°C.

Cytospin slides with 15.000 mDC were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.1% saponine (Merck, Darmstadt, Germany). After blocking with 0.1% saponine, 10% normal mouse serum (CLB, Amsterdam, The Netherlands) and 10% normal human plasma (Sanquin, Amsterdam, The Netherlands), slides were stained for 5 hours with anti-HLA-DR-APC (clone L243, BD) and anti-HBsAg-FITC (Acris Antibodies GmbH) at 4°C. After PBS wash, slides were sealed with Vectashield/DAPI (Vector Laboratories Inc., Burlingame, United States). Signals were captured with a Zeiss Laser Scanning Microscope 510 Meta JNI.

Expression of cell surface molecules on mDC

Isolated mDC ($30x10^3$/200µl) were matured in the presence of HBV, HBsAg or the appropriate controls. After 24 hours, cells were harvested and stained with combinations of anti-BDCA1-PE (Miltenyi Biotec), anti-CD80-FITC (clone MAB104, Immunotech, Marseilles, France), anti-CD86-APC (clone 2331, BD), anti-HLA-DR-PerCP (clone 243, BD) and anti-CD40-APC (clone 5C3, BD). Cells were analyzed by flow cytometry with corresponding isotype-matched control antibodies to determine background staining.

Cytokine production by mDC

Isolated mDC ($40x10^3$ cells/200µl) were stimulated in culture medium containing synthetic double-stranded poly[I:C] RNA (20 µg/ml; Sigma-Aldrich, St. Louis, MO) and recombinant human IFN-γ (1000 U/ml; Strathmann Biotech) in the presence of HBV, HBsAg or the appropriate controls. Supernatants were harvested after 24 hours. TNF-α and IL-10 levels were determined by ELISA from Biosource International (Nivelles, Belgium) and IL-12p70 was determined by ELISA from Diaclone (Besançon, France).

T cell stimulatory capacity of mDC

T cell stimulatory capacity of mDC was determined in an allogeneic mixed lymphocyte reaction (MLR). Isolated mDC were matured in the presence of HBsAg, HBV or the appropriate controls in culture medium containing IL-1β and TNF-α in a 96-well culture plate ($10x10^3$ or $5x10^3$/200 µl). After 24 hours, culture medium was removed and untouched isolated $CD3^+$ T cells (Miltenyi Biotec) from a buffy coat were added ($1.5x10^5$/200 µl) and cultured for six days. As a positive control, T cells were stimulated with phyto-haemagglutinin (PHA; 5 mg/ml; Murex, Paris, France) or anti-CD3 (100 ng/ml; clone UCHT1, eBioscience, San Diego, CA) and anti-CD28 (1 µg/ml; clone CD28.2, eBioscience). Supernatant was harvested at day 5 to determine IFN-γ production by ELISA (U-CyTech, Utrecht, The Netherlands). During the last 16 hours of a 6 day culture period, 0.5 mCi [^{3}H]thymidine (Amersham, Little Chalfont, UK) was present per well to determine T cell proliferation. All measurements were performed in triplicate. In a subset of experiments, the percentage of regulatory T cells was determined at day 5 by Foxp3 staining according to the manufacturer's protocol. Briefly, T cell surface markers were stained with a combination of anti-CD25-PE (clone M-A251, BD) and anti-CD4-PerCP (clone SK3, BD). The cells were fixed and permeabilized with fixation/permeabilization and permeabilization buffer (eBioscience) and anti-Foxp3-APC (clone PCH101, eBioscience) was present during permeabilization. Cells were analyzed by flow cytometry and $CD4^+CD25^{high}Foxp3^+$ cells were defined as regulatory T cells.

Statistical analysis

All experimental conditions were paired analyzed against their controls. Data on co-stimulatory molecules and cytokine expression were analyzed using the non-parametric Wilcoxon Signed Ranks Test with two-tailed p-values. Comparison of the data from MLR and IFN-γ production

was performed after logarithmic transformation of raw data. A mixed linear model with subject specific random effects was used with the Satterthwaite approximation method for correction of degrees of freedom. This method allows inclusion of all individual values obtained from triplicate measurements. Data from cytokine expression and MLR are normalized and given as percentages of the appropriate control. Data from expression of co-stimulatory molecules are expressed as mean±SEM. A p-value of <0.05 was considered statistically significant.

RESULTS

HBsAg is internalized by mDC

Although HBV is not capable to replicate within mDC, HBV particles and HBV DNA are detected on the surface or within DC [11-13,16]. To investigate whether HBsAg is also taken up by mDC, isolated mDC (Fig. 2A) were cultured in the presence of rHBsAg. Intracellular FACS analysis showed an increase of HBsAg uptake over time, increasing from 4±1% after 2 hours, to 27±2% after 18 hours. Control mDC incubated at 4°C showed no increase in HBsAg positivity, indicating that HBsAg internalization is an active process (Fig. 2B,C). To confirm the uptake of HBsAg, confocal microscopy was performed on cytospin slides of mDC incubated with rHBsAg or CHO control lysates. HBsAg was detected within the cytoplasm of mDC (Fig. 2D) and the percentage of HBsAg positive mDC increased to 12±1% after overnight culture (data not shown). No staining was detected in cells incubated with CHO control lysate.

HBsAg inhibits the upregulation of co-stimulatory molecules on mDC

Freshly isolated mDC from peripheral blood express only low levels of the co-stimulatory molecules CD40, CD80 and CD86. TNF-α/IL-1β-induced maturation results in a strongly increased expression of these co-stimulatory molecules (Fig. 3A-C) that is required for proper T cell activation. Since mDC are capable of internalizing HBsAg, the effect of HBsAg on mDC maturation was studied. CHO derived rHBsAg significantly inhibited upregulation of CD86 and also the upregulation of CD40 and CD80 tended to be inhibited. Next to the recombinant proteins, also patient serum-derived HBsAg was examined for its effect on mDC maturation. Both subtypes *ad* and *ay* of serum-derived HBsAg significantly inhibited the upregulation of CD40 and CD86 ($p<0.05$). Like the subviral protein HBsAg, purified HBV particles, which are also known to bind and be taken up by mDC, significantly inhibited the upregulation of CD40 and CD80 during TNF-α/IL-1β induced maturation compared to control conditions (Fig. 3D-F). Neither HBsAg, nor HBV did affect cell viability (data not shown).

HBV but not HBsAg inhibits the production of IL-12 after in vitro stimulation

Next to co-stimulatory molecules as important players in T cell activation, also cytokines produced by DC influence their T cell stimulatory capacity and their ability to polarize T cells. To

study the effect of HBsAg and HBV on the cytokine expression profile of mDC levels of IL-10, TNF-α and IL-12p70 were determined after 24 hr maturation in the presence or absence of HBsAg or HBV. Non-stimulated mDC did not show detectable levels of cytokine production (data not shown), whereas activated mDC clearly produced IL-10, TNF-α and IL-12 within 24 hr. No difference in expression levels of these cytokines was observed in the presence of serum derived or recombinant HBsAg (Fig. 4A-C). Also HBV particles did not influence the production of IL-10 and TNF-α (Fig. 4A,B). Interestingly, the presence of HBV particles induced a significant 4-fold reduction in the production of IL-12 (p=0.028, Fig. 4C,D).

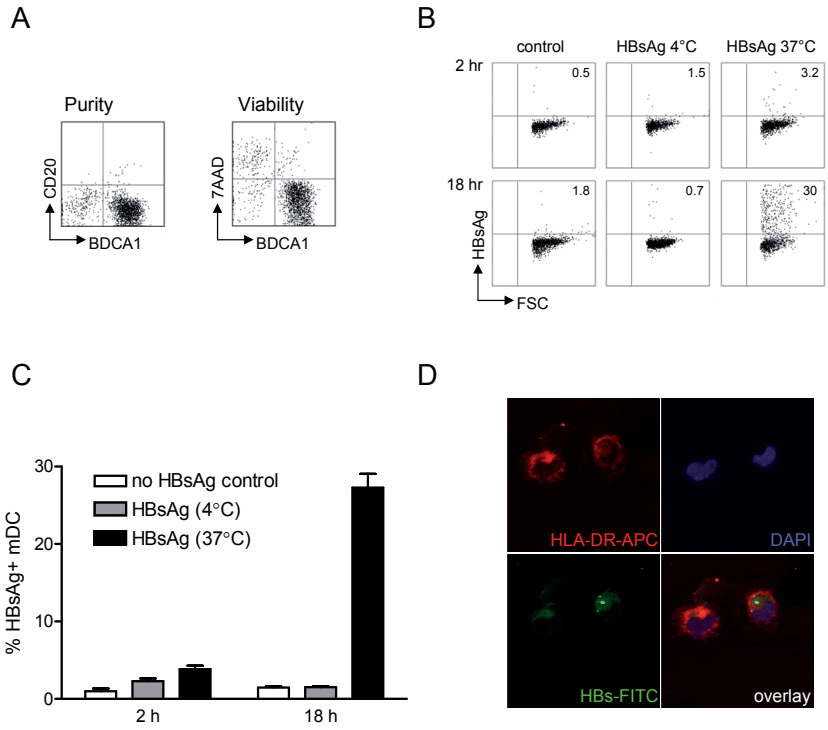

Fig. 2 HBsAg is actively internalized by mDC.
(A) Phenotypic analysis of isolated mDC. Purity and viability were checked by anti-CD20-FITC and 7AAD staining respectively. (B, C) mDC were incubated in the presence of GM-CSF with or without recombinant HBsAg during 2-18 hr at either 4°C or 37°C. After incubation, mDC were washed and HBsAg uptake was determined by flow cytometry. Data demonstrate a representative experiment (B) and the quantification of 6 independent experiments with mDC from different donors (mean±SEM) (C). (D) Cytospins of mDC cultured in the presence of HBsAg were stained for HLA-DR and HBsAg as described in Materials and Methods. Cell nuclei were visualized with DAPI. Two representative mDC were selected by confocal microscopy, one HBsAg positive and one negative.

Both HBV and HBsAg inhibit the T cell stimulatory capacity of mDC

Since both the expression of co-stimulatory molecules and the production of cytokines like IL-12 play a major role in T cell activation by DC, the effects of HBsAg and HBV on the T cell

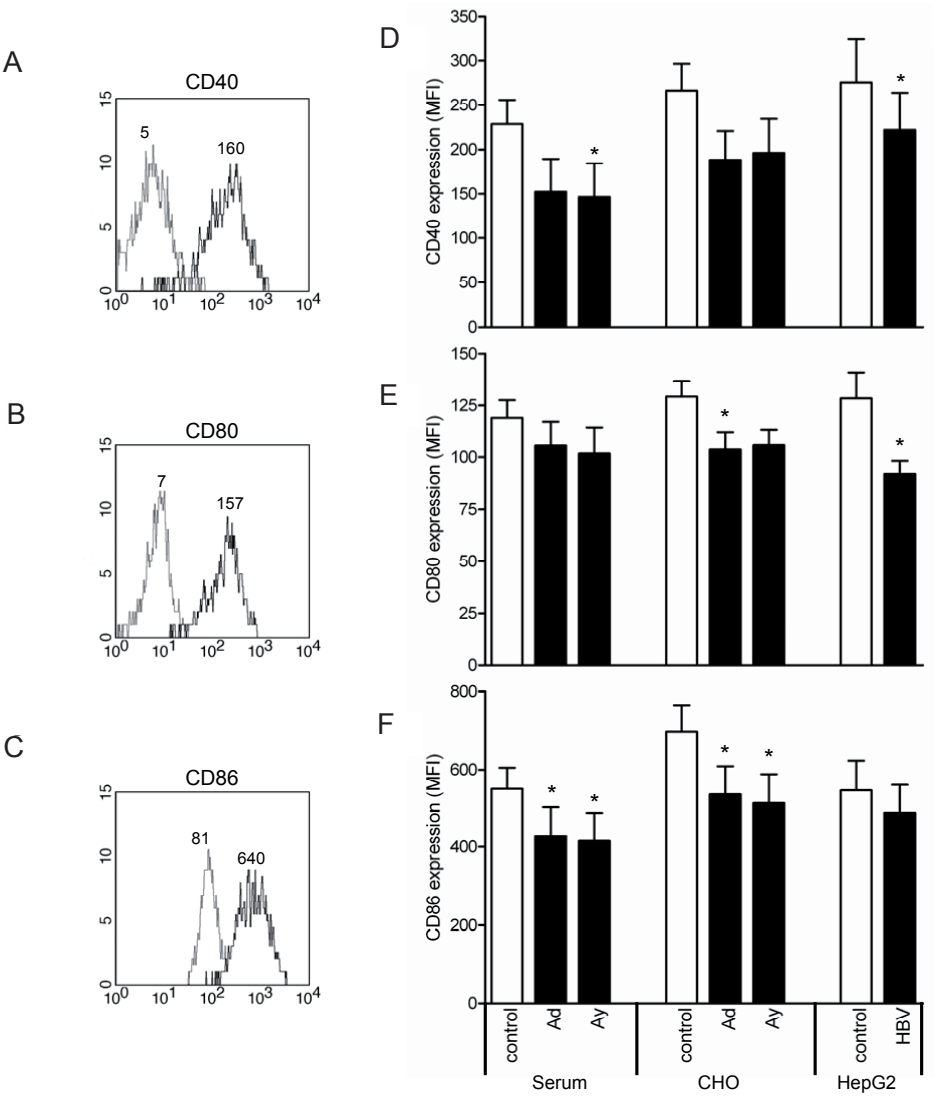

Fig. 3 HBsAg and HBV inhibit upregulation of co-stimulatory molecules during mDC maturation.
MDC were activated with TNF-α and IL-1β during 24 hr, in the presence of 1 μg/ml patient serum derived or CHO recombinant HBsAg (subtype ad and ay), HBV particles (MOI of 100) or the appropriate controls. The expression levels of co-stimulatory molecules CD40, CD80 and CD86 were determined by flow cytometry. (A, B, C) Mean fluorescence intensities of untreated mDC before (grey line) and after maturation (black line) of a representative experiment. (D, E, F) Quantification of the mean fluorescence intensities of CD40, CD80 and CD86 expressed on mDC matured in the presence of HBsAg and HBV, or the appropriate controls (n=8, mean±SEM, *p<0.05).

stimulatory capacity of mDC were determined in an allogeneic MLR. Matured non-treated mDC induced T cell proliferation which varied between different donors (range 4215-40977 cpm; ratio mDC:T = 1:15). Maturation of mDC in the presence of serum-derived HBsAg subtype *ad* reduced the T cell proliferation approximately 1.7-fold (Fig. 5A,B). Likewise, the T cell proliferation

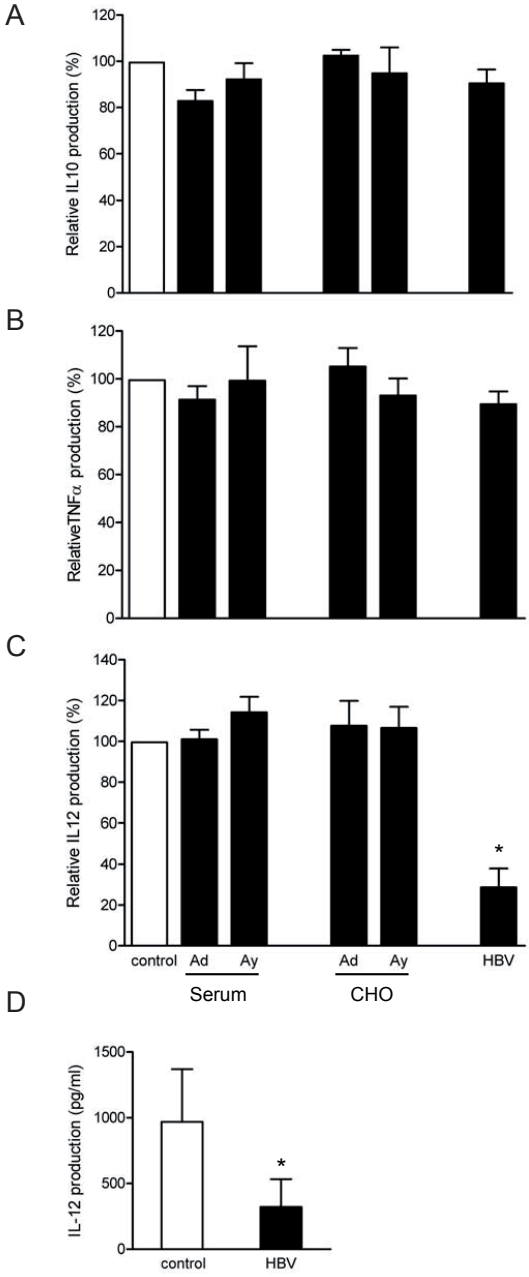

Fig. 4 HBV but not HBsAg reduced IL-12 production by mDC.

MDC were stimulated with poly(I:C) and IFN-γ in the presence of 1 μg/ml patient serum derived or CHO recombinant HBsAg (subtype ad and ay), HBV particles (MOI of 100) or the appropriate controls. After 24 hr, supernatants were harvested and examined for the production of IL-10 (A), TNF-α (B) or IL-12 (C, D). (A, B, C) Normalized data derived from 6 independent experiments with different donors are shown as mean±SEM percentage compared to appropriate control. (D) Mean IL-12 production of stimulated mDC of 6 donors in the absence or presence of HBV particles. *$p<0.05$.

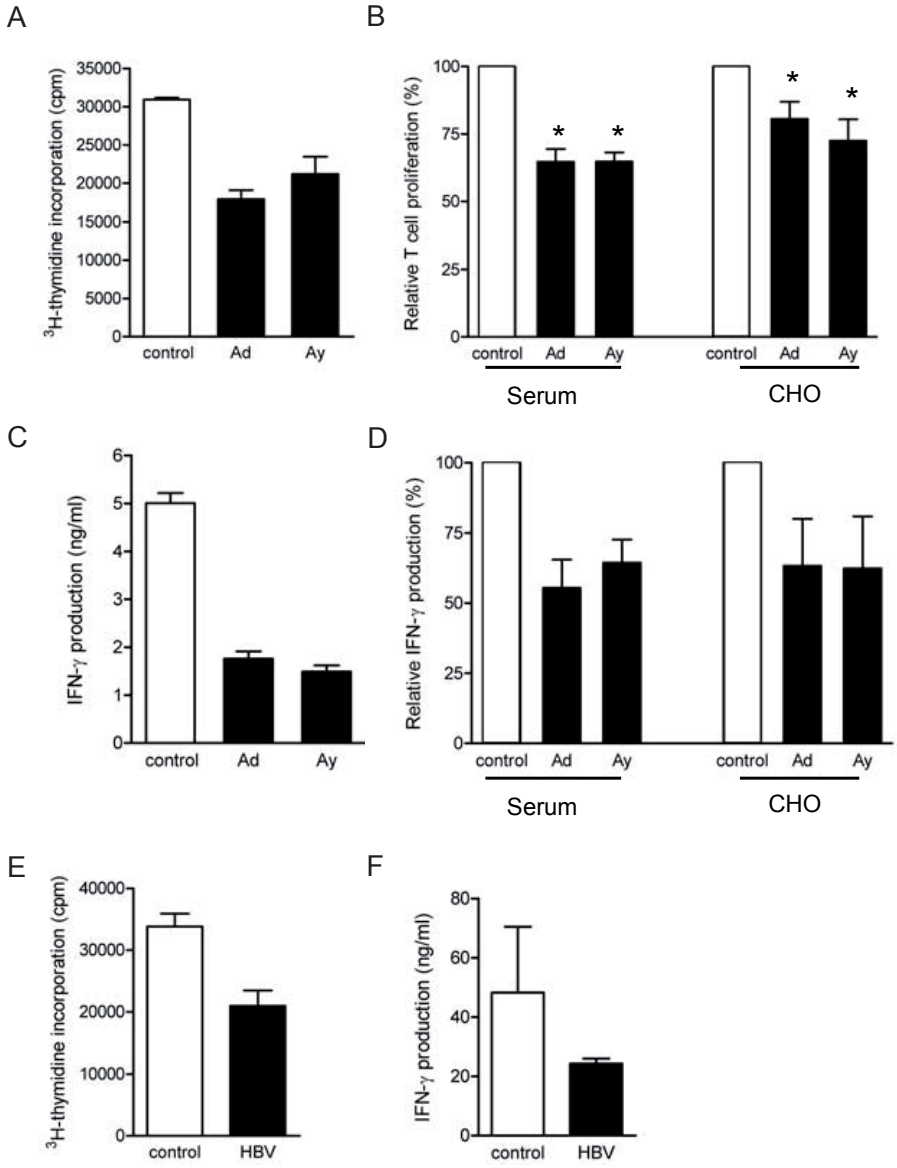

Fig. 5 HBsAg and HBV inhibit antigen presentation capacity of mDC.

MDC were activated for 24 hr with TNF-α and IL-1β in the presence of 1 μg/ml HBsAg or HBV particles (MOI of 100) or the appropriate controls and cultured for 6 days with allogeneic T cells. (A) Representative experiment of the effect of serum derived-HBsAg on the T cell stimulatory capacity of mDC, measured by 3H-thymidine incorporation in proliferating T cells (ratio mDC:T cells = 1:15) (B) Normalized data derived from 6 independent experiments with mDC isolated from different donors are shown as mean±SEM percentage compared to appropriate control. (C) Representative experiment of IFN-γ production after 5 days of co-culture of HBsAg-treated mDC and allogeneic T cells (ratio mDC:T cells = 1:15). (D) Normalized IFN-γ production in a subset of the same experiments as in (B) was determined and shown as mean±SEM percentage compared to appropriate control (n=4). (E, F) Representative experiment of the effect of HBV particles on the T cell stimulatory capacity of mDC measured by T cell proliferation (E) and IFN-γ production (F). *$p<0.05$

was inhibited in the presence of HBsAg subtype *ay*. Comparable to serum-derived HBsAg, recombinant HBsAg significantly reduced the T cell stimulatory capacity compared to control conditions (mean reduction: HBsAg *ad* 35±5% p=0.012, HBsAg *ay* 35±3% p=0.034, rHBsAg *ad* 22±7% p=0.007 and rHBsAg *ay* 33±9% p=0.0006; Fig. 5B). This effect was dose-dependently regulated and already observed with an HBsAg concentration of 0.1 µg/ml. HBsAg-induced inhibition reached a plateau at a concentration of 1 µg/ml.

Next to T cell proliferation, IFN-γ produced during these MLRs was measured in the supernatants after 5 days of culture. Non-treated mDC induced IFN-γ production which ranged between 3.7 and 71 ng/ml. HBsAg-treated mDC reduced induction of IFN-γ production by T cells to the same extent as observed for proliferation (Fig. 5C,D). HBsAg inhibited neither PHA nor anti-CD3/anti-CD28 induced T cell proliferation, indicating HBsAg has no direct effect on T cell proliferation (data not shown). HBV particles reduced the T cell stimulatory capacity of mDC to a similar extent as HBsAg as demonstrated by the significantly reduced T cell proliferation (mean reduction 34±6%, p<0.0001 Fig. 5E). Furthermore, HBV-treated mDC reduced the induction of IFN-γ production by T cells by approximately 2-fold (Fig. 5F). Thus, HBV as well as HBsAg inhibit mDC maturation and function, thereby giving rise to more tolerogenic mDC.

HBV and HBsAg-treated mDC do not induce regulatory T cells

One of the possible mechanisms by which HBsAg and HBV-treated mDC can reduce T cell proliferation is the induction of regulatory T cells, which can actively suppress T cell responses. The induction of this T cell subset during MLRs was analyzed by the expression of the regulatory T cell-specific transcription factor Foxp3 [22]. After 5 days of culture with non-treated mDC, approximately 2,5% of $CD4^+$ T cells could be defined as regulatory T cells ($CD4^+CD25^{high}Foxp3^+$, Fig. 6). No difference in regulatory T cell numbers was observed when T cells were stimulated

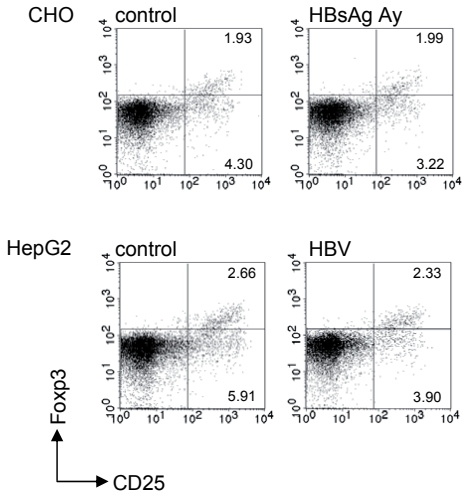

Fig. 6 HBV and HBsAg-treated mDC do not induce regulatory T cells.
MDC were activated for 24 hr with TNF-α and IL-1β in the presence of 1 µg/ml HBsAg or HBV particles (MOI of 100) or the appropriate controls and cultured for 5 days with allogeneic T cells. The percentage of regulatory T cells within the CD4+ cell fraction was determined by intracellular FACS staining. The number in the upper right quadrants indicates the percentage of regulatory T cells (CD4+CD25highFoxp3+). Representative dot blots of triplicate measurements of two independent experiments are shown.

with HBsAg or HBV-treated mDC. Interestingly, both HBsAg and HBV-treated mDC showed less T cell activation as indicated by the lower percentage of $CD4^{+}CD25^{high}Foxp3^{-}$ T cell subset (Fig. 6).

DISCUSSION

The liver and peripheral blood of HBV infected individuals can reach levels of 10^{9}-10^{10} infectious HBV particles per ml and 100 µg/ml of circulating HBsAg, which allows multiple interactions between the virus and DC. The present study demonstrates that myeloid DC actively internalize HBsAg and that the presence of either purified HBsAg or intact viral particles during mDC maturation gives rise to mDC with a significantly less immunogenic phenotype and function as demonstrated by the reduced expression of co-stimulatory molecules and decreased T cell stimulatory capacity.

Due to their pivotal role in anti-viral immunity, viruses, such as HBV, might have evolved to interfere with the function of DC to escape the host immune response. It has been demonstrated that DC of chronic HBV patients display a less immunogenic function compared to DC of healthy controls [9-11,23]. This DC impairment was recently challenged by a study of Tavakoli [24]. However, in that study only five to eight chronic HBV patients were included in the functional assays and the experiments were performed with cryopreserved instead of freshly isolated material, which may have strong impact on the function. HBV particles and HBsAg reduced the immunogenicity of mDC *in vitro*, which indicates a possible immune escape mechanism of HBV by the virus itself and/or by the production of subviral particles, i.e. HBsAg. The similar tolerogenic effects of HBsAg and HBV could easily be explained by the fact that the HBV envelop consists mainly of HBsAg. No major difference was observed between native patient serum derived HBsAg and CHO recombinant HBsAg although the latter contains not only S but also M forms of HBsAg. HBsAg subtypes *ad* and *ay* are very similar proteins according to charge and protein folding [25,26] and indeed showed comparable results in all assays. These data suggest that HBsAg, either as subviral particles or as part of the viral envelop, is at least partially responsible for the impaired mDC function observed in chronic HBV patients. This is in line with data from a HBsAg/HLA-A2 transgenic mouse model showing that high serum levels of HBsAg were able to induce T cell tolerance [27]. Whether HBsAg can also be detected on or within mDC of chronic HBV patients *in vivo* needs to be addressed in future studies.

Recombinant yeast-derived HBsAg has been shown to interact with monocytes through the LPS binding protein and the LPS receptor CD14, resulting in diminished LPS-induced monocyte activation. Using differentiated THP-1 cells as a model for monocytes, HBsAg was shown to reduce the LPS-induced TNF-α production through interference with the activation of ERK-1,2 and JNK-1,2 kinases [28,29]. The lack of effect of HBsAg on TNF-α production by mDC in the present study might be explained by differences between cell types and stimuli.

The direct effect of HBsAg on monocytes might be the explanation for the impaired function of DC generated from monocytes of chronic HBV patients [11]. In addition, the presence of HBV during the generation of monocyte-derived DC resulted in moDC with a reduced T cell stimulatory capacity and reduced IL-12 production [12]. Although in the latter study this might be more an effect of HBV on monocytes than on DC, also in the present study HBV was able to reduce IL-12 production by DC. Since IL-12 is a strong Th1 polarizing cytokine, the inhibitory effect of HBV on IL-12 production might partially explain the impaired Th1 response observed in chronic HBV patients. This hypothesis is supported by the fact that a 5-log reduction in viral load induced by the nucleoside analogue adefovir, strongly increased the capacity of mDC of chronic HBV patients to produce IL-12 [23]. In the latter study, also the T cell stimulatory capacity of mDC increased, which might be the result of a decreased viral load as well as decreased HBsAg levels in adefovir treated patients.

The additional tolerogenic effect of HBV over HBsAg with regard to the IL-12 production might be explained by the presence of the preS1 antigen on the viral envelop of HBV particles, which is not present in purified HBsAg, or by the presence of capsid protein or viral DNA. Nevertheless, both HBsAg and HBV diminished the T cell stimulatory capacity of mDC. Whether the reduction of co-stimulatory molecules on mDC could completely explain their decreased T cell stimulatory capacity, or that additional pro-inflammatory signals are decreased and/or regulatory signals are increased by HBsAg and HBV remains to be elucidated.

It has been previously reported that peripheral blood of chronic HBV patients contains an increased proportion of regulatory T cells compared to both individuals who have resolved their HBV infection and healthy controls [30]. This observation is apparently not caused by the effect of HBV and HBsAg on mDC, since both HBV and HBsAg-treated mDC did not significantly induce regulatory T cells in an allogeneic MLR. The observed reduction in T cell outgrowth and T cell activation as defined by high expression of CD25 might therefore be the result of T cell anergy or deletion induced by HBV and HBsAg-treated mDC.

The results of the present study might seem in contrast with several studies showing activation of DC after HBsAg-loading or during vaccine therapy, but these studies either added cytokines or uric acid to induce DC activation [31,32], or used an alum adjuvant containing HBsAg vaccine [33]. Of note, even though the routinely used HBsAg vaccination contains aluminium salts as an immune stimulatory adjuvant, about 5% of all HBV-vaccinated individuals do not develop a protective immune response, which is still not understood [34]. Therefore, it seems that only in the presence of immune activating stimuli a proper response to HBsAg could be induced, although even under this condition some individuals fail to develop an HBsAg-specific response. Further proof of the low immunogenicity of HBsAg is derived from studies on HBV-specific $CD8^+$ T cell populations: in contrast to HBV core-specific $CD8^+$ T cells which are associated with viral control, HBV envelop-specific $CD8^+$ T cells are characterized by an altered, HBV tolerant, phenotype. In addition, the frequency of HBV envelop-specific $CD8^+$ T cells is very low and they can only occasionally be found in the setting of high levels of HBV replication [35,36].

Next to HBV and HBsAg, serum of a subgroup of chronic HBV patients also contains HBeAg that could, in theory, also contribute to impaired mDC function. HBeAg has been described to be involved in T cell tolerance in a mouse model [37] but the exact function of HBeAg is still controversial [38]. The presence of either baculo- or *Escherichia coli*-derived recombinant HBeAg did not lead to a change in mDC function (data not shown).

In conclusion, these results show that both HBV particles and purified HBsAg have an immune modulatory capacity and may directly contribute to the dysfunction of mDC in chronic HBV patients. The direct immune regulatory effect of HBV and circulating HBsAg particles on the function of DC can be considered as part of the mechanism by which HBV escapes immunity. Successful immunotherapy of chronic HBV patients should therefore aim first on reduction of viral load and viral antigens in order to restore the immune system and thereafter on boosting the immune system to clear the chronic infection.

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CHAPTER 4

Branched oligosaccharide structures on HBV prevent interaction with both DC-SIGN and L-SIGN

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ABSTRACT

Hepatitis B virus (HBV) is a DNA virus that infects the liver as primary target. Currently, a high affinity receptor for HBV is still unknown. The dendritic cell specific C-type lectin DC-SIGN is involved in pathogen recognition through mannose- and fucose containing carbohydrates leading to the induction of an anti-viral immune response. Many glycosylated viruses subvert this immune surveillance function and exploit DC-SIGN as a port of entry and for *trans*-infection of target cells. The glycosylation pattern on HBV surface antigens (HBsAg) together with the tissue distribution of HBV would allow interaction between HBV and DC-SIGN and its liver-expressed homologue L-SIGN. Therefore, a detailed study to investigate the binding of HBV to DC-SIGN and L-SIGN was performed. For HCV, both DC-SIGN and L-SIGN are known to bind envelope glycoproteins E1 and E2. Soluble DC-SIGN and L-SIGN specifically bound HCV virus like particles, but no interaction with either HBsAg or HepG2.2.15 derived HBV was detected. Also, neither DC-SIGN nor L-SIGN transfected Raji cells bound HBsAg. In contrast, highly mannosylated HBV, obtained by treating HBV producing HepG2.2.15 cells with the α-mannosidase I inhibitor kifunensine, is recognized by DC-SIGN. The α-mannosidase I trimming of N-linked oligosaccharide structures thus prevents recognition by DC-SIGN. On the basis of these findings, it is tempting to speculate that HBV exploits mannose trimming as a way to escape recognition by DC-SIGN and thereby subvert a possible immune activation response.

INTRODUCTION

Hepatitis B virus (HBV) infects the liver as primary target, resulting in the majority of cases in self-limiting acute hepatitis. Nevertheless, more than 350 million people are chronically infected with HBV worldwide [1]. Despite the high incidence of infection, a cellular receptor for HBV entry is still unknown. Several putative binding factors have been described for the HBV surface antigens, such as human serum albumin [2], asialoglycoprotein receptor [3], heparin [4] and mannose binding lectin [5], but their exact role in HBV attachment and uptake remains unclear [6].

HBV is a DNA virus, consisting of a core particle enveloped by small (S), middle (M) and large (L) surface antigens, generally referred to as HBsAg. All three surface antigens contain a common S domain, both M and L proteins contain a preS2 domain and L exclusively contains a preS1 domain [7]. The liver and peripheral blood of HBV infected individuals can reach levels of 10^9-10^{10} infectious particles per ml. In addition, HBsAg is secreted from infected hepatocytes as spherical subviral particles and filaments, which can accumulate up to 100 µg/ml in peripheral blood [7,8]. S is the main component of both HBV virions and HBsAg subviral particles, while M and L are highly enriched on HBV virions. Post-translational modifications of the surface antigens are crucial for HBV life cycle; myristoylation of the preS1 domain is essential for infectivity [6,9], while inhibition of N-glycosylation of the preS2 domain prevents secretion of viral particles [10,11]. Recently, post-translational N-glycosylation of the preS1 domain has been reported as well and although it seems dispensable for HBV morphogenesis it might be involved in viral attachment [12].

The first step in clearance of a viral infection involves recognition of the virus by the innate immune system, mediated through host pattern recognition receptors (PRR) such as Toll-like receptors and C-type lectins [13,14]. C-type lectins recognize highly conserved pathogen-derived carbohydrate structures, leading to internalization, antigen processing and presentation to T cells [14]. A prototypical member of the C-type lectin family is the dendritic cell-specific lectin DC-SIGN [15], which recognizes a broad range of glycosylated pathogens through mannose- or fucose-containing carbohydrates, including HIV-1, hepatitis C virus, Ebola virus, Dengue virus, measles virus, human herpesvirus 8, SARS coronavirus, cytomegalovirus, *Mycobacterium tubercolosis, Helicobacter pylori, Streptococcus pneumonia* and *Neisseria meningitidis* [16-18]. The outcome of this interaction is pathogen-dependent. Many of these DC-SIGN-binding pathogens have evolved to subvert the immune surveillance function of DC-SIGN for their own benefit and re-direct the internalization route to non-lysosomal compartments for protection and transmission to target cells [16]. Some pathogens can also modulate adaptive immune responses through interaction with DC-SIGN; e.g. *Mycobacterium tuberculosis* exploits DC-SIGN to escape immune surveillance by inhibition of the immunostimulatory function of dendritic cells [16,19].

It is well established that envelope glycoproteins E1 and E2 of Hepatitis C virus (HCV) interact with both DC-SIGN [15] and its homologue L-SIGN [20] expressed on sinusoidal endothelial cells in liver and lymph nodes. For HCV virus-like particles, DC-SIGN interaction leads to efficient capture, internalization and transport to non-lysosomal compartments within immature dendritic cells, thereby protecting the virus from degradation [21,22]. Similarly, HCV virus-like particles interact with L-SIGN expressed on liver sinusoidal endothelial cells (LSEC) *in situ*, and are targeted to non-lysosomal early endosomes in L-SIGN transfected Raji cells [22]. DC-SIGN and L-SIGN are also thought to play an important role in HCV viral dissemination by transferring HCV from the circulation to hepatocytes, the main HCV target cells [23].

Several reports have shown the presence of HBV on or within dendritic cells [24-27], suggesting the involvement of a dendritic cell-specific receptor such as DC-SIGN. Patient-derived HBsAg is preferentially internalised by human LSEC in a mixed culture with human hepatocytes (K. Esser and U. Protzer, unpublished data) – as it has also been reported for duck HBV [28] - implying a potential interaction between HBV and L-SIGN. The HBV glycosylation pattern together with the cellular localization prompted us to investigate the possible role of both DC-SIGN and L-SIGN in binding of HBV.

MATERIALS AND METHODS

Antibodies and viral glycoproteins

The following antibodies were used: DC-SIGN and L-SIGN specific antibody AZN-D2 [29]; mouse anti-DC-SIGN conjugated with fluorescein isothiocyanate (FITC) (clone DCN46; BD Biosciences, San Jose, CA); mouse anti-HCV E2 (4H6B2; Innogenetics, Ghent, Belgium); sheep anti-HBsAg preS1, mouse anti-HBsAg preS2 and biotinylated human anti-HBsAg (F-9H9-E, all kind gifts of R. Heijtink, Erasmus MC, Rotterdam, The Netherlands); rabbit anti-HBsAg-FITC (Acris Antibodies GmbH, Hiddenhausen, Germany); peroxidase-conjugated goat anti-human immunoglobulin G1 (Jackson Immunoresearch, West Grove, PA); peroxidase-conjugated rabbit anti-FITC (Dako, Glostrup, Denmark); peroxidase-conjugated goat anti-mouse (Caltag; Carlsbad, CA); peroxidase-conjugated streptavidin (Dako).

Recombinant HBV surface antigen HBsAg (containing both S and preS2 domains) purified from transfected Chinese Hamster Ovary (CHO) cells was kindly provided by M. van Roosmalen (bioMérieux, The Netherlands). Yeast cell-derived HCV glycoproteins E1 and E2 reconstituted as virus like particles (VLP) were kindly provided by S. Depraetere (Innogenetics, Belgium).

HepG2.2.15-derived HBV

HepG2.2.15 cells [30] were grown until confluence in Williams' E medium (Gibco, Paisley, UK) supplemented with 5% fetal calf serum (FCS, Hyclone, Logan, UT). Secreted HBV and subviral particles were concentrated from the medium as described before [27], to a final concentration

of 0.96x10E9 HBV particles/ml. To enrich for HBV over subviral particles, HepG2.2.15 culture supernatants were loaded on a PBS equilibrated 1 ml bed volume Hi Trap™ Heparin HP column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). After fifteen column volumes PBS wash, HBV particles were eluted in 20 ml elution buffer (350 mM NaCl, 20 mM Tris/HCl, pH 7.4) and collected in elution fractions of 2 ml at a flow rate of 1 ml/min. Elution fractions were dialyzed against PBS. As a negative control, the same procedure was followed with supernatant from untransfected HepG2 cells. The elution fractions were assessed for the presence of HBV particles with L, M and S-specific ELISA, by capturing HBV with anti-preS1, anti-preS2 and anti-S specific antibodies respectively. Bound HBV was detected with a biotinylated human anti-HBsAg (F-9H9-E). Quantification of HBV particles was done by COBAS® TaqMan HBV Test (Roche Diagnostics GmbH, Mannheim, Germany) after viral DNA isolation using the High Pure System Viral Nucleic Acid Kit (Roche Diagnostics). To generate highly mannosylated HBV, HepG2.2.15 cells were treated with the α-mannosidase I inhibitor kifunensine (5 days, 20 µg/ml; Calbiochem, Darmstadt, Germany). Untreated HepG2.2.15 cells and kifunsine treated HepG2 cells served as negative controls. The final concentrations of highly mannosylated and native HBV were 1.4x10E8 and 2.2x10E8 HBV particles/ml, respectively.

Cells

Stable Raji transfectants expressing wild-type DC-SIGN or L-SIGN were generated as previously described [31, 32]. Monocyte-derived dendritic cells (moDC) were cultured from monocytes in the presence of IL-4 and GM-CSF (500 and 800 U/ml, respectively; Schering-Plough, Brussels, Belgium). At day 6, the phenotype of the cultured moDC was confirmed by flow cytometric analysis [22]. Expression levels of DC-SIGN and L-SIGN on transfected Raji cells and moDC were determined by flow cytometry with the DC-SIGN and L-SIGN specific AZN-D2 antibody.

Plant lectin ELISA

Heparin purified, glycan modified or native HepG2.2.15-derived HBV were captured on enzyme-linked immunosorbent assay (ELISA) plates (Maxisorb, Nunc, Roskilde, Denmark) with mouse anti-preS2. Recombinant HBsAg (0.5 µg/well), HCV VLP (0.25 µg/well) and mannan (0.25 µg/well; Sigma-Aldrich, St. Louis, MO) were coated directly onto the plate for 18 hours at 4°C. After blocking with 1% bovine serum albumin for 1 hr at 37°C, the following biotinylated plant lectins were added for 2 hr at room temperature at a concentration of 5 µg/ml: Con A (*Concavalin A*; recognizes α-glucose and α-mannose), GNA (*Galanthus nivalis* agglutinin; α-mannose), WGA (Wheat germ agglutinin; N-acetylglucosamine), PNA (*Arachis hypogaea* agglutinin; β-galactose, N-acetylgalactosamine), RCAII (*Ricinus communis* agglutinin; β-galactose, N-acetylgalactosamine) or LTA (*Lotus tetragonolobus* agglutinin; fucose; all from Sigma Aldrich [33]). Lectin binding was detected using peroxidase-conjugated streptavidin and absorbance was measured at 450 nm.

Recombinant DC-SIGN-Fc binding ELISA

The DC-SIGN-Fc binding assay was performed as previously described [34]. In short, recombinant HBsAg (0.5 µg/well), HepG2.2.15-derived HBV and HepG2 controls were captured on Maxisorb ELISA plates (Nunc) with mouse anti-preS2. HCV VLP (0.25 µg/well) were coated directly on the plates. After blocking with 1% bovine serum albumin for 30 min at 37°C, soluble DC-SIGN-Fc was added and bound DC-SIGN was detected after incubation with peroxidase-labelled anti-human immunoglobulin G1 antibody. Specificity of DC-SIGN-Fc binding was determined by blocking with either mannan (100 µg/ml; Sigma-Aldrich) or EGTA (10 mM; Sigma-Aldrich). To assess the coating efficiency, HBsAg and HBV were detected with biotinylated human anti-HBsAg (F-9H9-E) and HCV VLP were detected with mouse anti-HCV E2 (4H6B2). HBV concentrated by centrifugation or purified by heparin column gave similar results in DC-SIGN-Fc binding assays.

Soluble DC-SIGN/L-SIGN lysate ELISA

Raji DC-SIGN/L-SIGN transfectants were lysed for 4 hours at 4°C in lysis buffer (1% NP40, 150 mM NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ in PBS) supplemented with EDTA-free protease inhibitors (Roche Diagnostics, Penzberg, Germany). HBsAg, HBV and medium controls were captured on ELISA plates with a mouse anti-preS2 antibody. HCV VLP were coated directly on the plates. After blocking with 5% bovine serum albumin for 30 min at 37°C, Raji DC-SIGN/L-SIGN lysates were added for 2 hrs at RT. Bound DC-SIGN/L-SIGN was detected with a FITC-conjugated DC-SIGN/L-SIGN specific antibody (clone DCN46) followed by a peroxidase-conjugated rabbit anti-FITC antibody. Specificity of binding was determined in the presence of mannan (100 µg/ml; Sigma-Aldrich). To assess the coating efficiency, HBsAg, HBV and HCV VLP were detected with specific antibodies. HBV concentrated by centrifugation or purified by heparin column gave similar results in soluble DC-SIGN/L-SIGN lysate ELISA.

Cellular DC-SIGN/L-SIGN bindings assay

Untransfected Raji cells, DC-SIGN or L-SIGN-transfected Raji cells and moDC were incubated with or without HBsAg (5 µg/ml) for 2 or 18 hours at 37°C and binding was measured by flow cytometry after intracellular staining with an anti-HBsAg-FITC antibody (Acris Antibodies GmbH). Uptake was compared to lectin-mediated binding of dextran-FITC (100 µg/ml, 40.000 MW; Molecular Probes, Invitrogen, Carlsbad, CA). Specificity of binding was determined by 30 min pre-incubation with mannan (100 µg/ml).

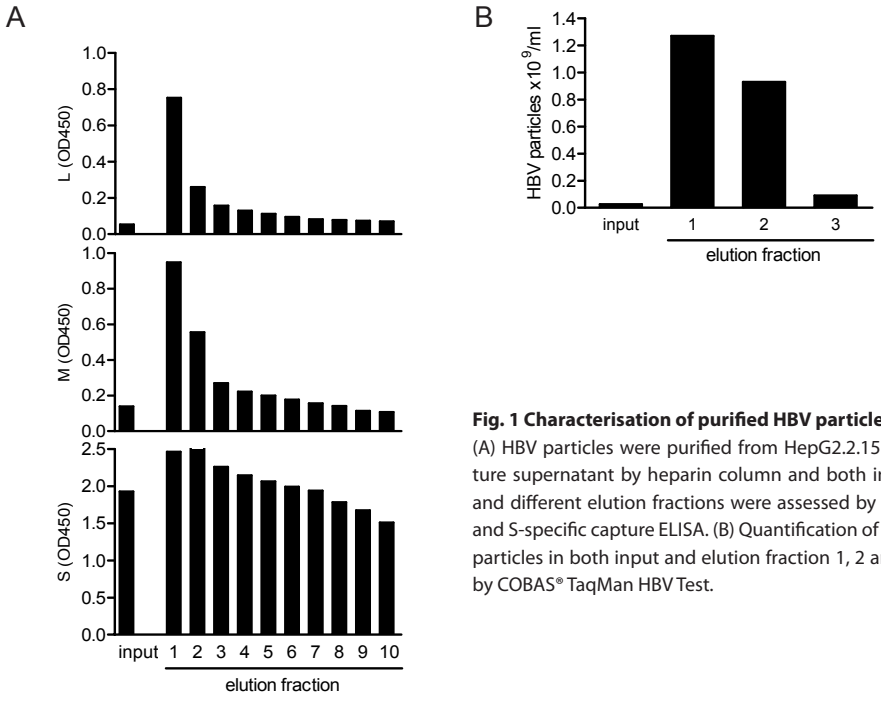

Fig. 1 Characterisation of purified HBV particles. (A) HBV particles were purified from HepG2.2.15 culture supernatant by heparin column and both input and different elution fractions were assessed by L, M and S-specific capture ELISA. (B) Quantification of HBV particles in both input and elution fraction 1, 2 and 3 by COBAS® TaqMan HBV Test.

RESULTS

Characterisation of purified HBV particles

Secreted HBsAg subviral particles outnumber the HBV virions at least 100-fold in both patient serum and culture supernatant of HepG2.2.15 cells [7]. To enrich for HBV virions, culture supernantant of HepG2.2.15 cells was fractionated over a heparin column and different elution fractions were assessed by HBV L, M and S-specific ELISA. The input fraction contained mainly the S protein, representing the relative high level of secreted spherical subviral particles (Fig. 1A). Elution fraction 1 and 2 however, were highly enriched for both M and L proteins confirming the increased level of HBV virions in these fractions. Quantification of HBV DNA of the input fraction and elution fraction 1-3 confirmed an almost 50-fold enrichment of HBV virions in fraction 1 (0,026 to 1,27x10^9 HBV particles per ml, Fig. 1B).

HBsAg and HBV do not interact with recombinant DC-SIGN-Fc

The interaction between DC-SIGN and recombinant HBsAg or HepG2.2.15-derived HBV was determined in a binding ELISA where coated HBsAg, HBV, HCV VLP or HepG2 medium controls were incubated with soluble recombinant DC-SIGN-Fc. The detected signal of the HepG2 medium control was never above background. Strikingly, DC-SIGN did not interact with HBsAg nor with whole virus particles, whereas DC-SIGN did interact with HCV virus like particles,

consisting of purified yeast recombinant E1 and E2 HCV envelope proteins (Fig. 2A and [22]). Specificity of binding was determined by blocking with either mannan, a yeast cell derived polycarbohydrate that competitively binds the carbohydrate binding site of mannose-specific lectins, or the calcium chelator EGTA, and both agents indeed reduced DC-SIGN binding to HCV to background level. Coating controls demonstrated that approximately equal amounts of HBsAg, HBV and HCV VLP were coated.

Based on their oligosaccharide specificity, several plant lectins were used in a glycan analysis of HBsAg and heparin purified HBV (Fig. 2B). Strong binding to Con A, GNA, WGA, PNA, RCAII and LTA demonstrated the presence of high and/or complex mannose, N-acetylglucosamine, galactose and fucose containing carbohydrate structures on both HBsAg and HBV. For DC-SIGN-glycoprotein interaction the high mannose and fucose containing oligosaccharide structures are most important [35]. Of note, carbohydrate structures on both HCV VLP and mannan mainly consist of mannose, as indicated by the exclusive binding to Con A and GNA.

HBsAg and HBV do not interact with soluble DC-SIGN from cell-lysates

To exclude the possibility that the lack of interaction between HBV and recombinant DC-SIGN was because of improper multimerization of DC-SIGN-Fc, binding of HBsAg and HBV to native DC-SIGN was studied with DC-SIGN derived from transfected Raji cells [31,32]. As described before, flow cytometric analysis of Raji transfectants showed high expression levels of DC-SIGN (Fig. 3A, [22]). In a bindings ELISA, coated HBsAg or HCV VLP were incubated with lysates of mock or DC-SIGN transfected Raji cells and bound DC-SIGN was detected with an anti-DC-SIGN antibody. DC-SIGN derived from Raji transfectants did not bind HBsAg nor whole HBV particles, whereas HCV VLP showed mannan-sensitive binding to DC-SIGN (Fig. 3B). Control incubation with anti-HCV and anti-HBsAg specific antibodies indicated equal amounts were coated. No background binding was observed for either HBsAg or HCV VLP using the lysate of untransfected Raji cells (data not shown).

HBsAg does not interact with cellular DC-SIGN

In addition to ELISA, HBsAg interaction with cellular DC-SIGN was also determined. DC-SIGN is highly expressed on moDC (Fig. 3A). Therefore, both Raji-DC-SIGN and moDC were incubated with HBsAg and binding was measured by flow cytometry. Neither DC-SIGN-positive Raji cells nor moDC interacted with HBsAg after 2 and 18 hr incubation (Fig. 3C and data not shown). In contrast, both DC-SIGN-transfected Raji cells and moDC efficiently internalised the DC-SIGN ligand dextran-FITC in a mannan-sensitive manner (Fig. 3C). Mock transfected Raji cells remained dextran-FITC negative (data not shown). Figure 3D summarizes the results of two independent binding assays.

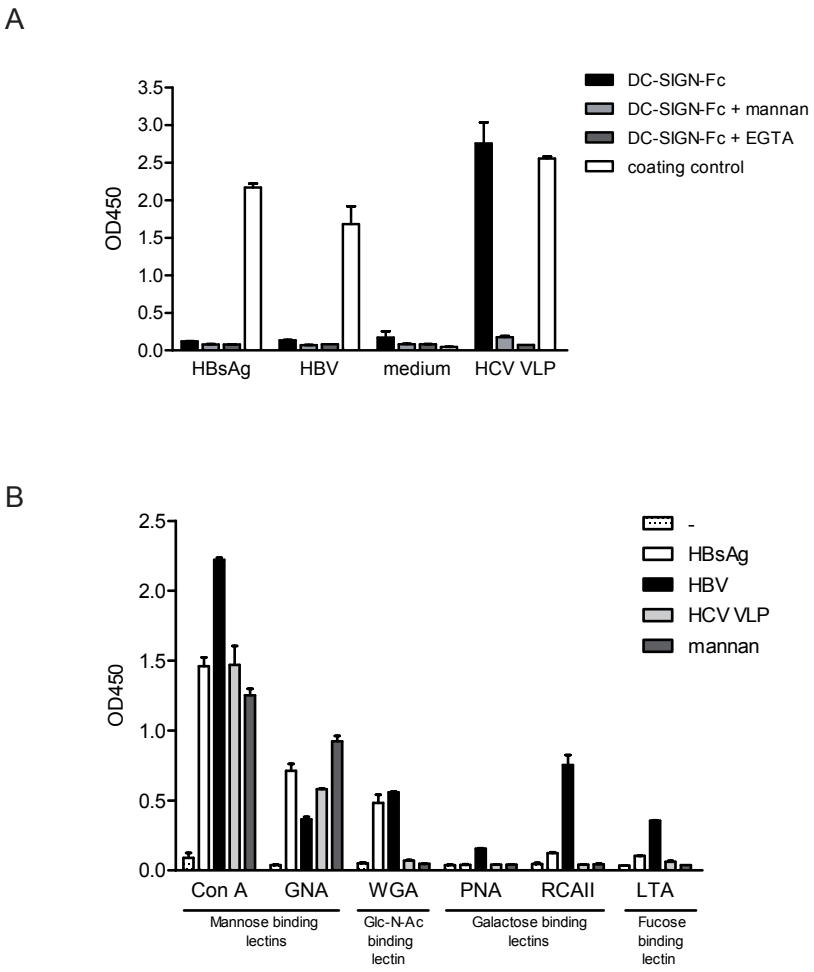

Fig. 2 HBV and its surface antigen HBsAg do not interact with recombinant DC-SIGN.
(A) DC-SIGN interaction with CHO-derived recombinant HBV surface antigen HBsAg and HepG2.2.15-derived HBV was determined in an Fc-based ELISA, as described in Methods. Supernatant of the non-virus producing cell line HepG2 was used as negative control and HCV VLP were used as a positive control. Specificity of binding was determined in the presence of mannan or the calcium chelator EGTA. To assess the coating efficiency, HBsAg, HBV and HCV VLP were detected with specific antibodies. Data are shown as mean±sd of duplicate measurements; one representative experiment out of four is shown. (B) Glycan analysis of HBsAg and HBV by plant lectin ELISA. For abbreviations and detailed oligosaccharide specificity of the indicated lectins see Methods.

HBsAg and HBV do not interact with soluble L-SIGN

In a similar manner, the interaction between cellular L-SIGN and both HBsAg and HBV was determined using L-SIGN-transfected Raji cells. Flow cytometric analysis showed L-SIGN expression levels equal to DC-SIGN expression (Fig. 4A, [22]). Soluble L-SIGN interacted neither with HBV nor with HBsAg in ELISA, whereas L-SIGN did interact with HCV VLP (Fig. 4B). Medium controls

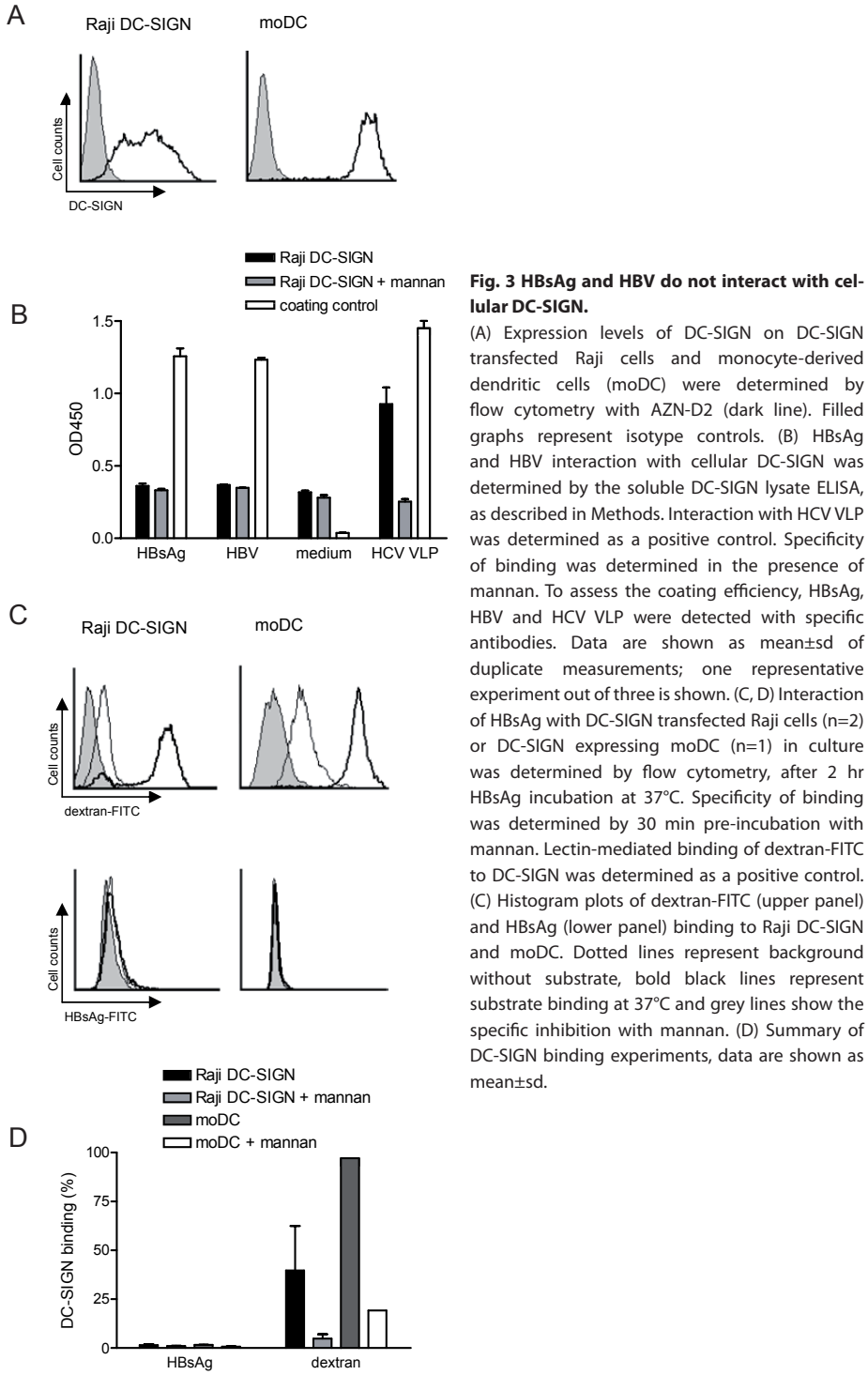

Fig. 3 HBsAg and HBV do not interact with cellular DC-SIGN.

(A) Expression levels of DC-SIGN on DC-SIGN transfected Raji cells and monocyte-derived dendritic cells (moDC) were determined by flow cytometry with AZN-D2 (dark line). Filled graphs represent isotype controls. (B) HBsAg and HBV interaction with cellular DC-SIGN was determined by the soluble DC-SIGN lysate ELISA, as described in Methods. Interaction with HCV VLP was determined as a positive control. Specificity of binding was determined in the presence of mannan. To assess the coating efficiency, HBsAg, HBV and HCV VLP were detected with specific antibodies. Data are shown as mean±sd of duplicate measurements; one representative experiment out of three is shown. (C, D) Interaction of HBsAg with DC-SIGN transfected Raji cells (n=2) or DC-SIGN expressing moDC (n=1) in culture was determined by flow cytometry, after 2 hr HBsAg incubation at 37°C. Specificity of binding was determined by 30 min pre-incubation with mannan. Lectin-mediated binding of dextran-FITC to DC-SIGN was determined as a positive control. (C) Histogram plots of dextran-FITC (upper panel) and HBsAg (lower panel) binding to Raji DC-SIGN and moDC. Dotted lines represent background without substrate, bold black lines represent substrate binding at 37°C and grey lines show the specific inhibition with mannan. (D) Summary of DC-SIGN binding experiments, data are shown as mean±sd.

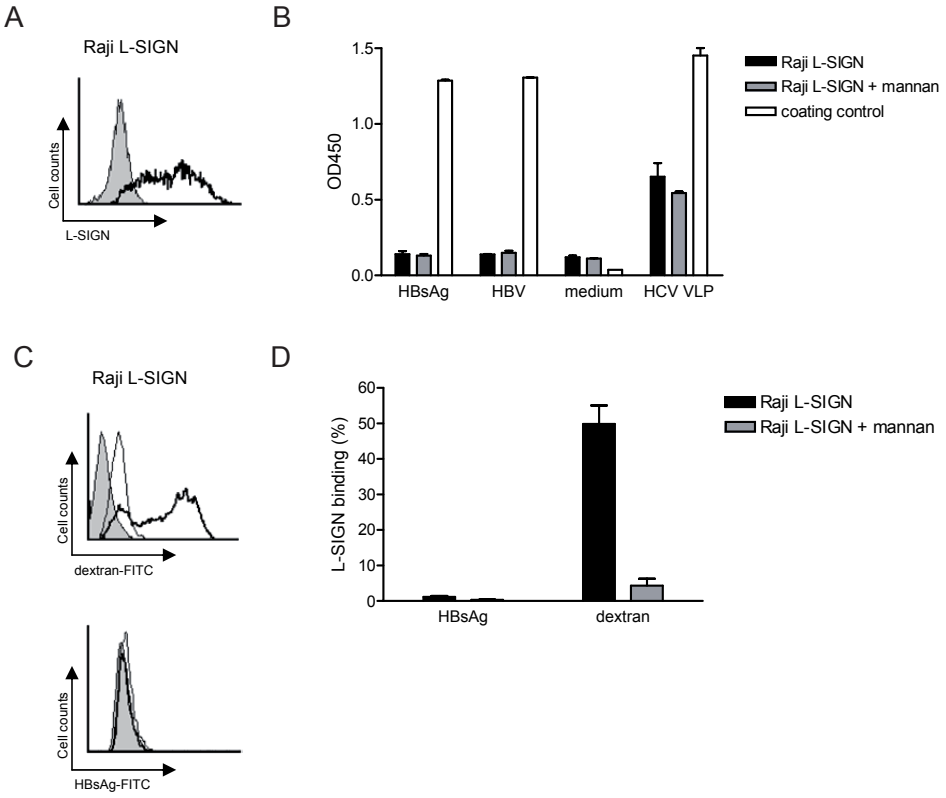

Fig. 4 HBsAg and HBV do not interact with cellular L-SIGN.
(A) Expression level of L-SIGN on L-SIGN transfected Raji cells was determined by flow cytometry with AZN-D2 (dark line). Filled graph represents isotype control. (B) HBsAg and HBV interaction with cellular L-SIGN was determined by soluble L-SIGN lysate ELISA. Data are shown as mean±sd of duplicate measurements; one representative experiment out of three is shown. (C, D) Interaction of HBsAg with L-SIGN transfected Raji cells in culture was determined by flow cytometry, as described in Fig. 3. L-SIGN binding of dextran-FITC was determined as a positive control. (C) Histogram plots of dextran-FITC (upper panel) and HBsAg (lower panel) binding to Raji L-SIGN. Dotted lines represent background without substrate, bold black lines represent substrate binding at 37°C and grey lines show the specific inhibition with mannan. (D) Results of two independent L-SIGN binding experiments, data are shown as mean±sd.

were always at background level. Moreover, cellular L-SIGN expressed by Raji transfectants did not bind to HBsAg, while it bound dextran-FITC in a mannan-sensitive manner (Fig. 4C, D).

DC-SIGN binds highly mannosylated HBV

Thus, neither HBsAg nor HBV interacts with DC-SIGN or L-SIGN, whereas both C-type lectins interact with HCV and other viruses such as HIV-1. These data suggest that the N-linked glycosylation of HBV is distinct from that of other viruses. To evaluate whether indeed native HBV glycosylation does not meet the requirements for DC-SIGN interaction, glycan modified HBV was generated by treating HBV-producing HepG2.2.15 cells with the α-mannosidase I inhibitor

kifunensine. Kifunensine causes the accumulation of $Man_{7-9}GlcNAc_2$ oligosaccharides on glycoproteins by inhibiting mannose trimming in the endoplasmic reticulum [36]. Carbohydrate analysis of glycan modified HBV indeed demonstrated an increased amount of mannose structures compared to native HBV, shown by a 2-fold increase in binding to the mannose-specific plant lectins Con A and GNA while binding to the N-acetylglucosamine-specific lectin WGA was unchanged (Fig. 5B). Control incubations with an anti-HBsAg specific antibody showed that similar amounts of native and highly glycosylated HBV were coated (Fig. 5A). The interaction between highly mannosylated HBV and DC-SIGN was studied by DC-SIGN-Fc-based ELISA (Fig. 5C). Strikingly, HBV binding to DC-SIGN was observed after kifunensine treatment of the virus producing cells, and the binding could be inhibited by both mannan and EGTA. The protein structure of HBV is therefore compatible with DC-SIGN binding, but the native glycosylation, that exclusively determines DC-SIGN interaction, is not. This observation also excludes the possibility that lack of DC-SIGN-HBV interaction is observed due to too low sensitivity of the DC-SIGN assays used in this study. Supernatant of untreated HepG2.2.15 and HepG2 cells showed no binding to DC-SIGN. Minor background binding was observed with supernatant of kifunensine-treated HepG2 cells, due to the accumulation of large amounts of highly mannosylated proteins in the medium during treatment. These data indicate that mannose trimming of the oligosaccharide structures present on HBV prevents interaction with DC-SIGN.

DISCUSSION

One of the key questions in HBV biology focuses on the attachment and entry mechanisms used by HBV to infect hepatocytes, the main target cells. Several putative binding receptors are proposed, but none of them are confirmed as high affinity receptors for HBV. Related to the issue of HBV entry is the way HBV reaches the hepatocyte: Is there direct contact between HBV in the circulation and hepatocytes, possibly through fenestrations in the LSEC [37]? Or are other cell types involved in HBV dissemination, similar to HIV transport by dendritic cells for trans-infection of T cells [31] and HCV capture by LSEC promoting infection of hepatocytes [38]?

Since HBV surface antigens are glycoproteins, the involvement of a C-type lectin receptor in viral recognition seems plausible. The best-characterised C-type lectins are the dendritic cell-expressed DC-SIGN and its LSEC-expressed homologue L-SIGN, both capable of recognizing a broad range of glycosylated pathogens, including viruses, bacteria and protozoa [16]. The fact that HBV has been found attached to both dendritic cells [24-27], and LSEC [28] leads to DC-SIGN and L-SIGN as likely candidate receptors.

The present study conclusively shows the lack of interaction between DC-SIGN and purified HepG2.2.15-derived HBV with its native glycosylation. Moreover, recombinant HBV surface antigens were recognized by neither DC-SIGN nor L-SIGN. Glycan analysis of both HBV and HBsAg demonstrated that lack of DC-SIGN binding was not due to aberrant glycosylation of virions and

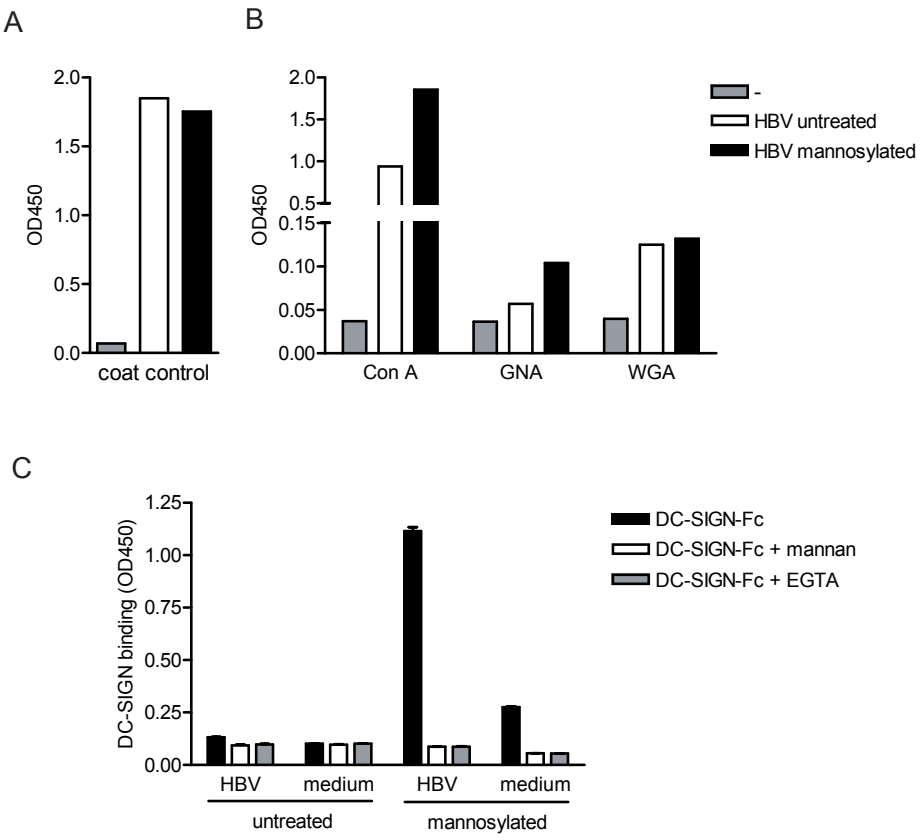

Fig. 5 DC-SIGN specifically binds highly mannosylated HBV.

Highly mannosylated HBV was generated by treating HBV producing HepG2.2.15 cells with the α-mannosidase I inhibitor kifunensine (20 µg/ml) for 5 days. Untreated HepG2.2.15 cells and kifunensine treated HepG2 cells served as negative controls. (A, B) Glycan analysis of untreated and mannosylated HBV by the mannose-specific lectins Con A and GNA and the N-acetylglucosamine-specific lectin WGA. In the coating control, the captured amount of untreated and mannosylated HBV is detected with an anti-HBS specific antibody. (C) The interaction of highly mannosylated HBV with DC-SIGN was studied by DC-SIGN-Fc ELISA, as described in Fig. 2. Specificity of binding was determined in the presence of mannan or EGTA. Supernatant of untransfected HepG2 cells is shown as medium control. Data are shown as mean±sd of duplicate measurements; one representative experiment out of three is shown.

glycoproteins used in this study, since the observed glycosylation pattern was consistent with previously reported complex-type carbohydrate structures on HBV [39]. Notably, while mannose and N-acetylglucosamine seem to be present in similar amounts on both HBV and HBsAg, glycan structures on virions are enriched for galactose, N-acetylgalactosamine and fucose. This difference in observed glycosylation pattern might be the result of O-glycans present on the preS1 domain [39].

DC-SIGN has been shown to interact with high mannose-containing glycans on various viruses, such as HCV, HIV and Ebola [16]. Even though data demonstrate that these viruses can exploit DC-SIGN for their own benefit, the majority of the captured virus is degraded and routed into the antigen presentation pathway [40-42], thereby allowing the induction of an anti-viral

immune response. Although HBV glycoproteins are involved in viral recognition, as they are known to bind mannose binding lectin and the asialoglycoprotein receptor, here it is shown that α-mannosidase I trimming of N-linked oligosaccharide structures prevents recognition by DC-SIGN. HBV is thus recognized by DC-SIGN as soon as one of the enzymes in the formation of complex glycans, α-mannosidase I, is inhibited and highly mannosylated virions are generated. Previous reports have shown the importance of HBV glycosylation for viral secretion [11,43] and indeed we observe a decrease in secretion of highly mannosylated versus native HBV, under similar culture conditions.

We cannot rule out that patient sera contain a subpopulation of these highly glycosylated viremia that would allow interaction with DC-SIGN. Preliminary data on HBV derived from several patient sera with a high viral load did not show binding to DC-SIGN-Fc (data not shown), thereby indicating that DC-SIGN may not be involved in recognition of HBV *in vivo* either. On the basis of our findings, it is tempting to speculate that HBV exploits mannose trimming as a way to escape recognition by DC-SIGN and thereby subvert a possible immune activation response. Further studies are needed to address this issue.

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CHAPTER 5

The mannose receptor acts as hepatitis B virus surface antigen receptor mediating interaction with intrahepatic dendritic cells

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ABSTRACT

Dendritic cells (DC) play a key role in anti-viral immunity. Direct interactions between DC and hepatitis B virus (HBV) may explain the impaired DC function and the ineffective anti-viral response of chronic HBV patients resulting in HBV persistence. Here, the interaction between HBV surface antigens (HBsAg) and DC and the receptor involved were examined by flow cytometry in blood and liver tissue of HBV patients. The *in vitro* data showed that the mannose receptor (MR) is involved in HBsAg recognition and uptake by DC. The presence of HBsAg-positive DC was demonstrated sporadically in blood, but frequently in the liver of HBV patients. Interestingly, a positive correlation was found between HBsAg positivity and MR expression level in both liver- and blood-derived DC. These data suggest that in HBV infected patients, MR-mediated interaction between HBsAg and DC and subsequent impairment of DC predominantly occurs at the main site of infection, the liver.

INTRODUCTION

In the majority of cases, infection with Hepatitis B virus (HBV) results in self-limiting acute hepatitis, due to a vigorous multi-specific T and B cell response [1]. However, worldwide more than 350 million people are chronically infected with HBV [2] as the result of a complex interaction between the replicating virus and an inadequate HBV-specific immune response. The underlying mechanism for this ineffective anti-viral immune response is not clear.

Dendritic cells (DC) are key players in the anti-viral immune response and form a bridge between innate and adaptive immunity [3]. They are equipped with specialized pattern-recognition receptors, including C-type lectins and Toll like receptors [4,5]. These receptors enable them to recognize and internalize pathogens resulting in antigen processing and presentation. The nature of the pathogen and the receptors involved in pathogen recognition determine the activation status of the DC, which in turn determines the quality of the immune response ranging from tolerance to immunity [6].

HBV primarily infects hepatocytes, but both *in vitro* and *in vivo* data indicate that the virus also interacts with DC. HBV DNA was demonstrated in isolated blood DC from chronic HBV patients [7-9]. Recently, we showed that HBV surface antigens (HBsAg) are internalized by blood-derived myeloid DC (mDC) *in vitro*, leading to impaired mDC function [10]. Also several *in vivo* studies describe the impaired function of mDC in chronic HBV patients [7,11,12]. This impaired mDC function was improved upon viral load reduction [12]. However, whether *in vivo* a direct immune regulatory effect of HBV on DC function exists, which receptor(s) are involved in this interaction and whether the impaired function of blood mDC could be explained by circulating HBV and/or HBsAg is not known. Several putative binding factors have been described for HBsAg, such as human serum albumin [13], asialoglycoprotein receptor [14] and mannose binding lectin [15], but their exact role in HBV attachment and uptake remains unclear [16]. Since HBsAg are glycoproteins [17], the involvement of a C-type lectin in viral recognition by DC seems plausible. DC are known to express both type I and type II C-type lectins, including DEC-205 (CD205), mannose receptor (MR; CD206) and DC-SIGN (CD209), but the expression of the different C-type lectin family members depends on the DC subset and differentiation/maturation state [5].

Most studies on pathogen interactions with human DC are performed with *in vitro* generated monocyte-derived DC, which exhibit functional and phenotypical features of, but are not identical to, circulating mDC. To unravel the possible interaction between HBV and mDC *in vivo*, we investigated the uptake of HBsAg, the receptor involved and the presence of HBsAg-positive mDC in blood and liver *in vivo*.

MATERIALS AND METHODS

Isolation of mDC from peripheral blood

All healthy controls gave written informed consent before blood donation. MDC ($BDCA1^+$) were isolated from healthy control blood using anti-BDCA1-PE and PE-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously [7]. MDC purity and viability were determined by flow cytometry (FACScalibur, Becton, Dickinson and Company (BD), Franklin Lakes, NJ). Gated on viable cells, the purity of isolated mDC ranged from 85 to 95%. MDC were cultured in RPMI 1640 (Bio Whittaker, Verviers, Belgium) supplemented with 10% FCS (Hyclone, Logan, UT), penicillin (50 IU/ml), streptomycin (50 µg/ml) and GM-CSF (500 U/ml; Leucomax, Novartis Pharma, Arnhem, The Netherlands).

Lectin-mediated endocytosis

MDC were directly used to study the endocytic capacity or first cultured for 18 hr, followed by 2 hr incubation in the presence or absence of dextran-FITC (100 µg/ml; 40.000 MW, Molecular Probes, Invitrogen, Carlsbad, CA). For HBsAg binding and uptake, mDC were incubated for 18 hr with recombinant HBsAg (2 µg/ml, kindly provided by M. van Roosmalen, bioMérieux, Boxtel, the Netherlands) [10]. Antigen uptake was stopped by cold wash in PBS containing 1% FCS and 0.02% NaN_3. HBsAg was stained by intracellular staining with anti-HBsAg-FITC (Acris Antibodies GmbH, Hiddenhausen, Germany) as described before [10] and cells were measured by flow cytometry. This protocol detects both surface bound and internalized HBsAg. Lectin-mediated endocytosis was inhibited by 30 min pre-incubation at 37°C with mannan (100 µg/ml; Sigma-Aldrich, St. Louis, MO), D-mannose, D-glucose, D-fructose, D-galactose, fucose, N-acetyl glucosamine, N-acetyl galactosamine (50 mM; Sigma-Aldrich), EGTA (20 mM; Sigma-Aldrich), neutralizing anti-MR (1-20 µg/ml; clone 19.2, BD) or neutralizing control antibody anti-DEC205 (10 µg/ml; clone MG38, AbD Serotec, Kidlington, UK). Negative controls were incubated without antigen or with antigen at 4°C.

Lectin expression

MDC were lysed in Trizol (Sigma-Aldrich) directly after isolation and after 18 hr of culture. RNA was precipitated with 75% ethanol and isolated using Micro RNAeasy silica columns (Qiagen, Venlo, The Netherlands). cDNA was prepared using AMV reverse transcriptase according to the manufacturer's protocol (Promega, Madison, WI). Real-time PCR (MyIQ, Biorad, Hercules, CA) was performed using primers for GAPDH (F: 5'-CCATGTTCGTCATGGGTGTG-3'; R: 5'-GGTGCTAAGCAGTTGGTGGTG-3'), DEC-205 (F: 5'-CCCTATGTCTGCAGGAAACCA-3'; R: 5'-TGCATCACAGCGGGTATCTG-3') and MR (F: 5'-gtcttgggccacaggtgaa-3'; R: 5'-aaggcgtttggatagccaca-3') with iQ SYBR Green supermix (Biorad) for 40 cycles and gene expression was calculated using the ΔΔCT method. Protein expression levels of MR and DEC-205 were determined by flow cytometry after staining with anti-BDCA1-PE (clone AD5-8E7,

Miltenyi), anti-CD20-PerCP (BD), anti-CD205-FITC (clone MG38, eBioscience, San Diego, CA) and anti-CD206-APC (clone 19.2, BD) in PBS containing 1% FCS and 0.02% NaN_3.

HBsAg staining on patient-derived blood and liver tissue

Blood and percutaneous needle liver biopsies were obtained from 14 chronic HBV patients and 9 non-HBV patients with other liver diseases as part of their diagnostic evaluation. Control patients were negative for markers of prior HBV exposure. The medical ethical committee of the Erasmus MC declared to have no objections against the use of excess patient material and all patients gave informed consent before inclusion. Patient characteristics are given in Table 1. Excess tissue from liver biopsy samples was collected in RPMI and grinded through a 70 µM nylon cell strainer (BD) to obtain a single cell suspension of mononuclear cells, which were stained with a combination of anti-BDCA1-PE, anti-CD14-PerCP (clone MOP9, BD), anti-CD20-PacificBlue (clone 2H7, eBioscience), anti-CD45-AmCyan (clone 2D1, BD) and anti-CD206-APC followed by intracellular staining with anti-HBsAg-FITC. Cells were measured by 8-color flow cytometry (FACSCanto II, BD) and analyzed with BD FACSDiva software. MDC were identified as $CD45^+CD14^-CD20^-BDCA1^+$ and assessed for the presence of HBsAg and expression of MR. Only measurements with an mDC population of at least 100 events were included; background HBsAg-staining in control non-HBV patients was never above 2%. Hepatocytes were gated on forward/sideward scatter and CD45 negativity and assessed for presence of HBsAg.

RESULTS

C-type lectin mediated endocytosis by mDC increases upon culture

Our previous study on HBsAg internalization by mDC showed an increased uptake over time [10], which might be mediated through C-type lectins considering the glycosylation pattern of HBsAg. Many reports describe C-type lectin expression and endocytic capacity of monocyte-derived DC, but little is known about blood-derived mDC. Therefore, the capacity of C-type lectin mediated endocytosis of mDC and the culture-induced change in endocytic capacity was determined with the model substrate dextran-FITC. Directly after mDC isolation, uptake after 2 hr culture in the presence of dextran-FITC is limited to approximately 15% mDC. Overnight culture in the presence of GM-CSF, followed by 2 hr dextran-FITC uptake, increased uptake to 36±5% positive mDC (Fig. 1A). As expected, uptake of dextran-FITC could be blocked by addition of mannan, which competitively binds the carbohydrate binding site of mannose-specific lectins. In addition, dextran-FITC uptake was strongly reduced by addition of D-mannose, D-glucose, D-fructose, fucose, N-acetyl glucosamine and the calcium chelator EGTA (Fig. 1B). Galactose and N-acetyl-galactosamine did not inhibit dextran-FITC uptake by mDC, indicating that e.g. the galactose-specific asialoglycoprotein receptor and macrophage galactose-type C-type lectin are not likely to contribute to lectin-mediated endocytosis by mDC.

Table 1 Patient characteristics

Patient #	Age (yr)	Sex	Liver Disease	Genotype	Serum Viral Load[a,b]	Serum ALT[c] (U/l)
1	30	M	HBV	D	$4.17*10^3$	38
2	45	M	HBV	D	$5.12*10^9$	155
3	32	M	HBV	D	$6.46*10^4$	78
4	26	M	HBV	C	$8.48*10^8$	444
5	55	M	HBV	A	$3.05*10^6$	58
6	29	M	HBV	D	$2.50*10^7$	77
7	31	M	HBV	ND	$4.34*10^3$	28
8	39	F	HBV	D	$7.16*10^5$	30
9	27	F	HBV	ND	$3.41*10^3$	49
10	40	M	HBV	ND	$2.25*10^{10}$	101
11	39	F	HBV	B	$1.57*10^9$	35
12	22	F	HBV	D	$2.14*10^3$	86
13	32	F	HBV	ND	$2.37*10^8$	46
14	28	F	HBV	ND	$2.27*10^4$	38
15	55	M	HCV	3	ND	160
16	64	F	HCV	1	$9.86*10^5$	182
17	48	M	HCV	1	$3.58*10^7$	16
18	42	M	LTx[d]	NA	NA	63
19	41	M	Other	NA	NA	43
20	45	M	HCV	4	ND	59
21	22	M	HCV	1	$2.55*10^7$	43
22	36	F	HCV	1	$8.17*10^6$	78
23	42	M	HCV	1	$2.84*10^6$	92

NA, not applicable; ND, not determined
[a]HBV DNA and HCV RNA are given in geq/ml
[b]Within 12 weeks of biopsy time point
[c]Within 2 weeks of biopsy time point
[d]Due to primary sclerosing cholangitis

C-type lectin expression on mDC

Since both HBsAg [10] and dextran-FITC uptake increased after overnight culture, the expression level of several C-type lectins on mDC was determined by real-time PCR before and after 18 hr of culture. No detectable signal over background was obtained for L-SIGN, ASGPR1 and Endo-180 (data not shown). ASGPR2A and B mRNA could be detected in mDC directly after isolation, while mRNA levels diminished after mDC culture (data not shown). In contrast to the high level of DC-SIGN mRNA in monocyte-derived DC ([18], data not shown), DC-SIGN mRNA could not be detected in mDC directly after isolation, although it was present at very low levels after 18 hr of culture (data not shown). Also MR and DEC-205 mRNA are known to be expressed by monocyte-derived DC ([18], data not shown). In contrast, MR mRNA levels were undetectable in freshly isolated mDC, but increased 8- to 32-fold during the 18 hr culture period (Fig. 2A,B). DEC-205 mRNA was already present at high levels in mDC directly after isolation and was 2-fold reduced after culture (Fig. 2A,B). Flow cytometric analysis also showed an increase

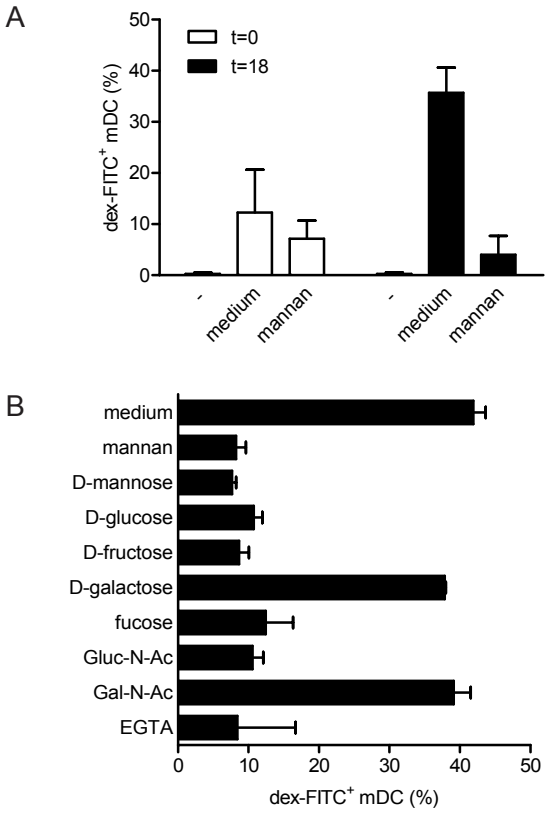

Fig. 1 Lectin-mediated endocytosis of dextran-FITC by mDC increased upon culture.
(A) MDC were analysed for dextran-FITC uptake, either directly after isolation or after O/N culture. Specificity was determined by 30 min pre-incubation with mannan. Blancs (-) represent background fluorescence in the absence of dextran-FITC. Data represent mean±sd of two independent experiments. (B) O/N cultured mDC were examined for dextran-FITC uptake after 30 min pre-incubation at 37°C with different sugars or EGTA. Data represent mean±SEM of three independent experiments.

of MR-positive mDC after 18 hr culture (Fig. 2C). DEC-205 was highly expressed on the mDC cell surface after isolation, but in contrast to the mRNA expression level, the protein expression level did not significantly change upon culture (Fig. 2D).

HBsAg internalization is mediated through the MR

The glycosylation pattern of HBsAg, and the increased HBsAg internalization combined with the increased MR expression upon mDC culture, indicated the MR as the most likely candidate for HBsAg uptake. The exact role of the MR in HBsAg internalization was studied by using mannan as inhibitor and more specifically, by blocking the receptor with increasing amounts of neutralizing anti-MR antibodies. Mannan significantly inhibited HBsAg uptake (Fig. 3A). Increasing amounts of neutralizing anti-MR antibody led to dose-dependent decrease of HBsAg uptake, with a maximum block using 10 µg/ml anti-MR (Fig. 3A). MR neutralization led to a significant 2-fold reduction of HBsAg uptake, compared to both medium control and presence of control antibody anti-DEC-205 (Fig. 3B; both $p<0.007$). No significant effect of neutralizing DEC-205 on HBsAg uptake was observed.

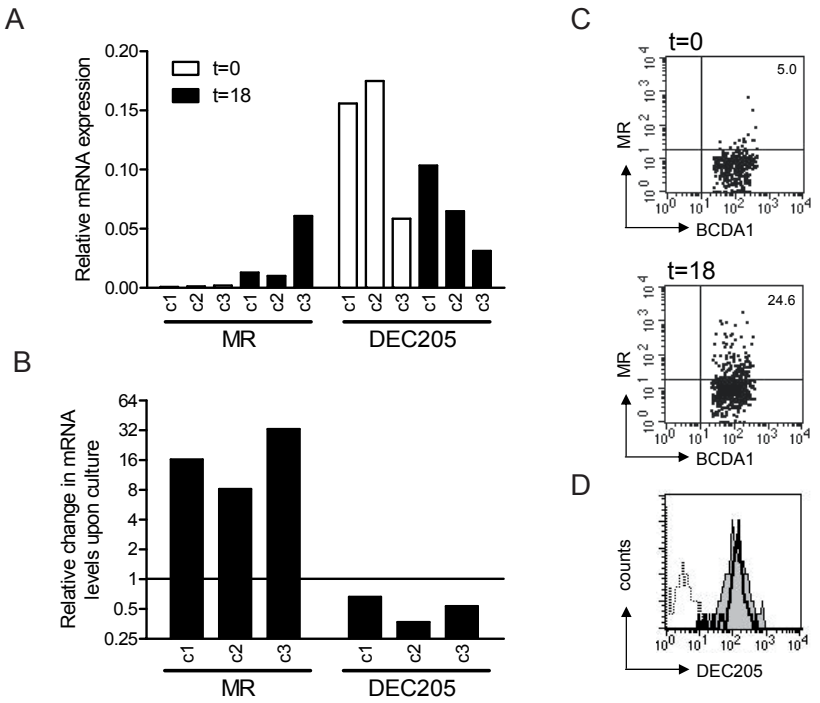

Fig. 2 Expression of C-type lectins on mDC.

(A) MR and DEC205 mRNA levels relative to GAPDH of mDC of three different donors (c1-3) directly after mDC isolation and after 18 hr culture. (B) Fold change in relative mRNA expression of MR and DEC205 upon culture of mDC of three different donors (c1-3). (C) Protein expression level of MR on the cell surface of CD20-BDCA1+ mDC before and after 18 hr culture. One representative experiment out of three independent experiments is shown. (D) Histogram analysis of DEC205 expression on mDC before (bold line) and after (filled graph) 18 hr culture. The dotted line represents isotype control. One representative experiment out of three independent experiments is shown.

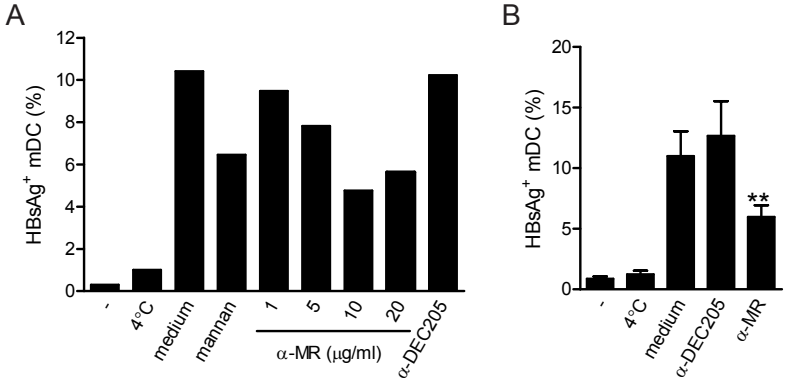

Fig. 3 *In vitro* HBsAg uptake by mDC is mediated through the MR.

HBsAg uptake after O/N culture was measured by flow cytometry. Specificity was determined by 30 min pre-incubation with mannan, increasing amounts of neutralizing anti-MR antibody, or control anti-DEC205 antibody. Negative controls were incubated without HBsAg (-) or with HBsAg at 4°C. Data demonstrate a representative experiment (A) and the quantification of eight independent experiments (B)

Intrahepatic mDC of chronic HBV patients contain HBsAg

To examine whether also *in vivo* mDC are capable of internalizing HBsAg, either as subviral particle or as part of the infectious virion, we studied the presence of HBsAg in blood- and liver-derived mDC of chronic HBV patients. Blood and liver biopsy samples of patients with liver diseases other then HBV were used as negative controls. Gating strategies to identify blood and intrahepatic mDC populations were similar; except for the FSC/SSC gate since intrahepatic mDC were a bit smaller and less granular than blood-derived mDC (Fig. 4). Two out of 14 chronic HBV patients clearly contained HBsAg-positive mDC in the circulation (patient# 7 and 9, Table 2), but in most patients blood-derived mDC were HBsAg-negative and HBsAg-staining did not significantly differ from control group (Fig. 5A). Strikingly, liver biopsies from 10 out of 14 HBV patients contained HBsAg-positive mDC, while no HBsAg positivity was detected in mDC of control liver biopsies. Paired analysis of blood- and liver-derived mDC of chronic HBV patients showed significantly higher amounts of intrahepatic HBsAg-positive mDC (Fig. 5A; 3.8±1% intrahepatic vs 1.4±0.3% blood-derived (mean±SEM) HBsAg–positive mDC; p=0.047). The number of HBsAg-positive mDC found in the liver varied between patients and positively correlated with the number of HBsAg-positive hepatocytes, the main target of HBV (Table 2, Fig. 5B, p=0.0017).

Table 2 HBsAg and MR levels of blood- and liver-derived myeloid DC and hepatocytes

Patient	Blood mDC			Liver mDC			Hepatocytes	
	Total events	HBsAg$^+$ (%)	MR$^+$ (%)	Total events	HBsAg$^+$ (%)	MR$^+$ (%)	Total events	HBsAg$^+$ (%)
1	630	0.2	0.3	2318	2.8	3.5	22692	18.3
2	254	1.3	0.0	310	7.1	20.3	17989	16.9
3	484	1.9	11.8	915	0.3	9.3	9468	3.1
4	340	1.2	1.2	1200	0.1	5.1	13628	1.0
5	310	1.0	1.6	1668	4.0	5.8	9107	26.3
6	756	0.8	1.6	166	4.8	12.7	10233	18.2
7	1055	3.1	1.7	635	0.8	4.7	7557	16.4
8	1143	1.0	1.0	1351	3.0	5.9	20609	24.9
9	421	3.8	4.3	2349	0.8	2.4	16720	14.3
10	1240	0.6	1.0	1168	1.9	2.3	15227	0.8
11	1736	0.6	1.2	160	14.4	14.4	1360	44.5
12	400	1.0	1.8	362	3.3	20.7	14045	3.2
13	416	1.9	2.2	727	5.0	12.5	16002	26.5
14	141	0.7	1.4	1393	5.5	5.6	12737	73.4
15	146	0.7	10.6	367	1.1	6.8	9323	0.1
16	730	1.9	3.3	858	1.3	4.2	20330	0.1
17	546	1.1	4.6	564	1.8	10.3	13391	0.1
18	1000	0.7	1.8	356	1.1	12.9	12734	0.2
19	576	0.2	5.7	1350	0.5	7.2	16689	0.3
20	311	0.3	1.6	230	1.3	18.3	8227	0.2
21	624	1.3	1	1641	1.5	2.6	16194	0.5
22	484	1.9	6.4	1343	0.3	5.2	20143	0.2
23	429	0.7	1.6	1849	0.5	3.7	11329	0.4

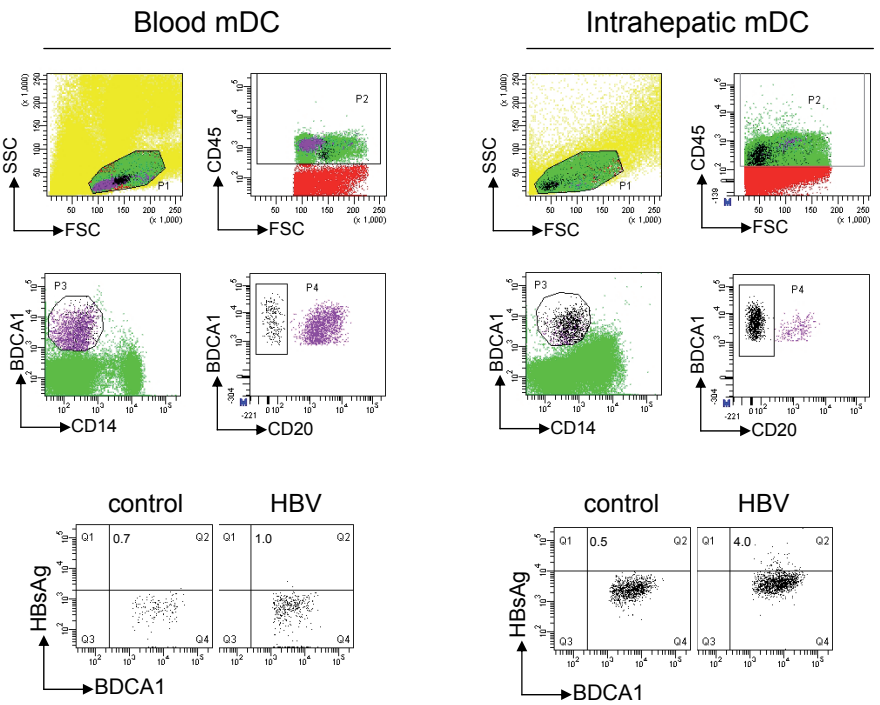

Fig. 4 Flow cytometric analysis of blood- and liver-derived mDC.
Blood and liver biopsy samples of patients with chronic HBV and control patients with non-HBV related liver diseases were measured by flow cytometry after staining for anti-CD45, anti-CD14, anti-CD20, anti-BDCA1 and anti-HBsAg. The left panel shows the mDC gating strategy of a representative blood sample; the right panel shows a representative liver biopsy sample. MDC are identified as CD45+CD14-CD20-BDCA1+, within this population the percentage HBsAg positive cells was determined as shown for a representative control (patient# 23) and HBV patient (patient# 5).

In vivo presence of HBsAg in mDC correlates with MR expression

Since HBsAg is abundantly present in both blood and liver of chronic HBV patients, the HBsAg concentration cannot explain the difference in HBsAg uptake between blood- and liver-derived mDC. Thus the ligand specificity and/or the endocytic capacity should differ between blood- and liver-derived mDC. The *in vitro* experiments demonstrated a key role for MR in HBsAg uptake, but upregulation of this receptor by cell culture was a prerequisite for HBsAg internalization. Therefore, the MR expression level of blood- and liver-derived mDC of chronic HBV patients was studied. Intrahepatic mDC showed a significantly 4-fold higher MR expression compared to peripheral blood-derived mDC (Fig. 6A; 8.9±1.7% vs 2.2±0.8% MR-positive mDC; p=0.034). When individual patients were studied, a significant positive correlation was found between HBsAg positivity and MR expression level in both liver- and blood-derived mDC (Fig. 6B).

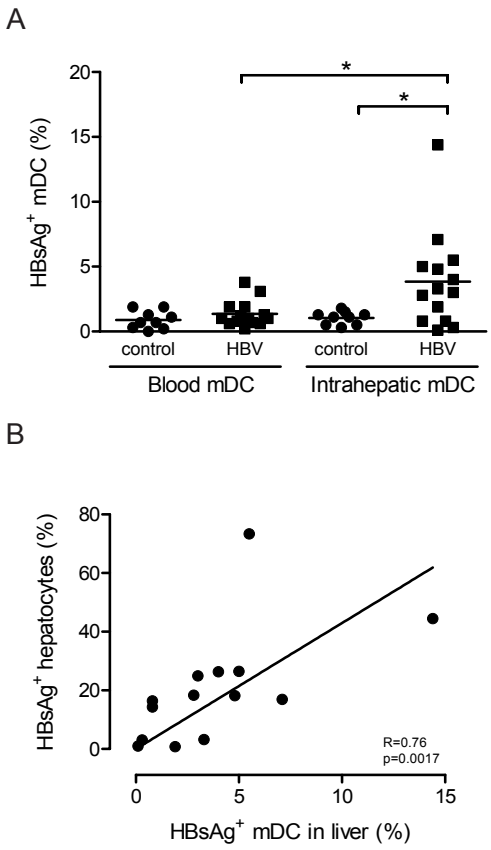

Fig. 5 HBsAg is mainly detected in liver-derived mDC.
(A) Blood- and liver-derived mDC populations of 14 chronic HBV and 9 control patients were assessed for presence of HBsAg (*p<0.05). (B) Liver biopsy samples were used to determine the presence of HBsAg-positive hepatocytes, gated on FSC/SSC and CD45 negativity. The correlation between presence of HBsAg in hepatocytes and intrahepatic mDC was determined with the Spearman test (r=0.76, p=0.0017).

DISCUSSION

DC are professional antigen-presenting cells with a critical role in the initiation and direction of anti-viral immune responses [3]. C-type lectins on DC recognize glycan structures expressed on pathogens, resulting in pathogen internalization followed by processing and antigen presentation on MHC molecules [5]. The present study provides insight in the level of interaction between mDC and HBsAg and the role of the MR in this interaction. We demonstrate that the MR is involved in the uptake of HBsAg by mDC *in vitro* and also show HBsAg-positive mDC in HBV infected individuals *ex vivo*. While only a minority of the chronic HBV patients contained HBsAg in blood-derived mDC, a significant number of liver-derived mDC of these patients contained HBsAg, which correlated with MR expression. These data demonstrate that HBV interacts with mDC *in vivo*, especially at the main site of infection, i.e. the liver, and point towards a crucial role for MR in this interaction.

Most circulating blood mDC were found to be negative for the MR, which was reflected by the lack of HBsAg *ex vivo* and the relative low capacity to take up dextran-FITC as a model substrate for lectin-mediated endocytosis. This low lectin-mediated endocytic capacity is in line with data of Kato *et al*. and in sharp contrast to the high dextran-FITC uptake by *in vitro* generated monocyte-derived DC [18]. Although it is known that expression of e.g. cell surface molecules such as C-type lectins varies [19], monocyte-derived DC are frequently used as a model for mDC. A clear example is the C-type lectin DC-SIGN: highly expressed on monocyte-derived DC but not present on circulating blood mDC, as shown before [19] and confirmed here by real-time PCR analysis. Culture of mDC in the presence of GM-CSF enhanced dextran-FITC uptake and sugar inhibition studies indicated that mannose-specific receptor mediated endocytosis mainly contributed to this binding. Real-time PCR combined with flow cytometric analysis on the expression of C-type lectins, indicated a role for the MR in binding and uptake of HBsAg by mDC. Blocking experiments with mannan and, more specifically, a neutralizing antibody against the MR confirmed the role of MR in HBsAg uptake by mDC *in vitro*.

The MR expression on the cell surface of cultured blood-derived mDC and intrahepatic mDC, but not on circulating mDC in whole blood, likely reflects the difference in differentiation state and endocytic capacity between mDC present in blood and peripheral tissue. Plausibly, mDC are recruited to the site of infection, acquire the MR on the cell surface as a result of migration-induced differentiation and subsequently internalize HBV and/or HBsAg. Whether MR expression is influenced by liver inflammation could not be determined, since control patients were also presented with liver inflammation, as indicated by the elevated alanine aminotransferase levels. A more definite answer could come from analysis of steady state livers, but without a clinical need to obtain a liver biopsy such material is rarely available.

The expression of HBsAg by infected hepatocytes in almost all biopsy samples taken from chronic HBV patients included in this study and the correlation between the level of HBsAg positive-hepatocytes and -intrahepatic mDC, not only indicates that the biopsy sample was taken at a site of active HBV infection, but also shows that the virus-DC interaction takes place at the site of infection.

Next to the MR, other classes of C-type lectins have been described to interact with HBsAg. Chong *et al*. have demonstrated that HBsAg can interact with soluble mannose-binding lectin leading to complement activation, which could contribute to viral clearance by enhanced phagocytosis [15] and may explain the potential contribution of mannose-binding lectin polymorphisms in HBV disease progression (reviewed in [20]). Furthermore, the asialoglycoprotein receptor, expressed by e.g. liver sinusoidal endothelial cells (LSEC) and hepatocytes, has been shown to interact with HBV virions through the preS1 domain of HBsAg [14]. Since LSEC are also known to express MR [21], the MR could play a role in the capture and transportation of HBV virions across the liver endothelium to infect adjacent hepatocytes, comparable to the model proposed by Knolle *et al.* [22]. Whether the MR could also play a role in the infection of hepatocytes is not known. In this study we observed that approximately 10% of hepatocytes

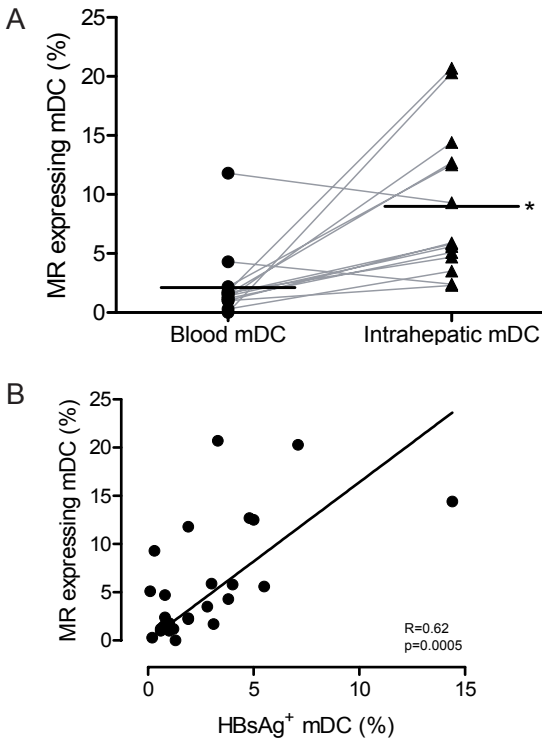

Fig. 6 Correlation between presence of HBsAg and MR expression level in mDC of chronic HBV patients.
(A) MR cell surface expression of blood- and liver-derived mDC of 14 chronic HBV patients was measured by flow cytometry (mean±SEM MR-positive mDC, *p<0.05). (B) The correlation between HBsAg positivity and presence of MR on blood- and liver-derived mDC was determined with the Spearman test (r=0.62, p=0.0005).

express the MR (data not shown), possibly contributing to the capture of HBsAg and/or HBV virions.

Although C-type lectin DEC-205 belongs to the same family as the MR and is highly expressed on mDC, it does not interact with HBsAg. A difference in ligand specificity is the obvious explanation, although DEC-205 ligands still need to be determined [23]. More surprisingly is the observed recognition of HBsAg by MR, but not by DC-SIGN [24]. Whereas most pathogens recognized by the MR also show interaction with DC-SIGN, e.g. Mycobacterium tuberculosis and HIV [25], the subtle differences between ligand specificity of DC-SIGN and MR, namely the recognition of complex mannose structures versus end-standing mannose residues [26], leads to major differences in HBsAg recognition.

In contrast to pathogen recognition by e.g. Toll like receptors, interaction with C-type lectins DC-SIGN and MR does not induce DC maturation [27]. Thus the MR mediated HBsAg uptake in the absence of a Toll like receptor signal may lead to HBV/HBsAg-specific tolerance, which could be further supported by the microenvironment of the liver. From unpublished observations we know that neither HepG2.2.15-derived HBV nor HBsAg are able to induce DC maturation. Moreover, both HBV and HBsAg can directly reduce the immunogenicity of DC *in vitro*, which indicates a possible immune escape mechanism of HBV by the virus itself and/or by

the production of HBsAg [10,28]. Although there are conflicting data published, several studies demonstrated an impaired DC function in chronic HBV patients, which could at least partially explain HBV persistence [7,9,11,12,29,30]. This immune regulatory effect of HBV is supported by the fact that an adefovir-induced viral load reduction strongly increased the capacity of mDC of chronic HBV patients to produce IL-12 and to stimulate T cell activation [12]. The finding that only two out of 14 chronic HBV patients demonstrated HBsAg-positive mDC in blood, is comparable to a study by Untergasser *et al*., showing HBV DNA positive mDC in one out of five patients [8]. In contrast, two other studies show HBV DNA in mDC of either half the patient population [7] or even in 10 out of 12 patients [9]. Diversity between patient cohorts included in each study might explain part; another important difference is the detection method of HBV.

In conclusion, the present study for the first times shows the involvement of the MR in binding of HBsAg. The immune competence of chronic HBV patients combined with the relative low level of interaction between HBV/HBsAg and mDC in peripheral blood indicates that HBV especially interferes with mDC function in the liver. Whether the MR is directly involved in the immune regulatory effects of HBV and HBsAg remains to be elucidated.

ACKNOWLEDGEMENT

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CHAPTER 6

Hepatitis B virus lacks immune activating capacity, but actively inhibits plasmacytoid dendritic cell function

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Submitted

ABSTRACT

Chronic Hepatitis B virus (HBV) infection is caused by inadequate anti-viral immunity. Toll like receptor (TLR) crosslinking in plasmacytoid dendritic cells (pDC) leading to interferon-α (IFN-α) production is crucial for effective anti-viral immunity. HBV infection lacks IFN-α induction in animal models and patients and chronic HBV patients display impaired IFN-α production by pDC. Therefore, the effect of HBV on the function of human pDC was examined *in vitro*. In contrast to influenza or HSV, HBV did not activate pDC nor induced cytokine production. Moreover, combined exposure to either CpG or Lox to trigger TLR9 or TLR7 respectively, demonstrated that HBV dose-dependently reduced TLR9-induced pDC function, whereas TLR7-induced pDC function was hardly affected. In line, HBsAg binding to pDC was strongly enhanced upon TLR9, but not TLR7 triggering. HBV inhibited CpG-induced upregulation of co-stimulatory molecules, production of IFN-α, TNF-α, IP-10 and IL-6 and pDC-induced NK cell function without affecting cell viability. The immune regulatory effect of HBV on purified pDC was even further enhanced by addition of monocytes to the cultures. Analyzing different HBV proteins revealed that HBeAg, but especially HBsAg is involved in suppression of pDC function. In line, HBV as well as HBsAg abrogated the CpG-induced mTOR-mediated phosphorylation of S6, subsequent phosphorylation of IRF7 and transcription of IFN-α genes.

Thus, HBV fails to activate pDC and inhibits TLR9-induced mTOR-mediated pDC function via a direct effect on pDC that can be further enhanced by monocytes, which may both contribute to HBV persistence. The findings that HBV is not only a weak inducer of innate immunity, but also actively interferes with pDC function may aid in the design of novel treatment strategies for chronic HBV.

INTRODUCTION

Hepatitis B virus (HBV) infects the liver as primary target and may elicit progressive liver injury leading to increased risk of developing liver cirrhosis, liver failure and liver cancer [1]. Chronic infection with HBV is the result of an ineffective anti-viral immune response towards the virus [1,2]. The exact mechanism by which HBV escapes immunity is still not known.

In general, the immune system is alerted and evokes a number of mechanisms that are aimed at eradicating the viral attack immediately following viral infection. The initial response to viral infection is the rapid release of type I interferons (IFN), IFN-α and IFN-ß, which is observed for most viruses studied [3]. These IFN enhance the first defense against viral infections and have several direct effects in modulating both innate and adaptive immune cells. Indications of the role of type I IFN during HBV infection are mostly based on studies in chimpanzees, since this is the only animal that can be infected with HBV. In sharp contrast to other viruses including Hepatitis C virus, chimpanzees infected with HBV showed a complete lack in the induction of type I IFN and in IFN-response genes during the early stages of infection [4]. It is difficult to study the early events of acute HBV infection in humans. Nevertheless, it was recently shown that type I IFN responses are also lacking in acute HBV patients [5]. Plasmacytoid dendritic cells (pDC) are the principal producers of type I IFN and play a central role in immune responses against viral infections [6,7]. PDC respond to viruses and other pathogens primarily through the recognition of pathogen-associated molecular patterns by two intracellular Toll-like receptors (TLR), TLR7 and TLR9, which recognizes single stranded RNA and unmethylated DNA motifs, respectively [8,9]. TLR-triggering activates pDC to rapidly produce high levels of type I interferons, but also other cytokines, including TNF-α and IL-6, and cell surface co-stimulatory molecules. In this way pDC exert a direct anti-viral effect by producing factors that inhibit viral replication, but they also activate natural killer (NK) cells and T lymphocytes allowing further priming and regulation of anti-viral immunity [6,10,11].

Circulating blood pDC numbers seem to be unaffected by HBV, but functional deficits in pDC from chronic HBV patients have been reported [12]. In these studies, impaired IFN-α production by pDC from chronic HBV patients was observed after stimulation with SAC [13], HSV [14], or DNA containing CpG motifs [15,16] as compared to healthy controls. Concerning blood-derived pDCs, viral DNA could be detected in a subset of chronic HBV patients but no evidence was found for viral replication in these cells [13,17]. The presence of HBV DNA in or on pDC *in vivo* indicates a direct interaction between pDC and viral particles. Moreover, additional interactions between pDCs and HBV-derived proteins present in circulation, such as HBeAg or HBsAg, which can reach levels of 100 µg/ml in peripheral blood [18], may also impact pDC function. Given the central role that these cells play in both innate and adaptive antiviral immune responses, understanding the mechanisms whereby pDC interact with and respond to HBV may provide fundamental insights into the regulation of HBV-specific immunity and the development of HBV chronicity.

The present study shows that HBV does not activate pDC, but inhibits pDC function via a direct effect on pDC that can be further enhanced by monocytes. Analyzing different HBV proteins revealed that HBeAg and especially HBsAg are involved in HBV-mediated suppression of pDC function by interfering with TLR9-induced S6 phosphorylation. These data demonstrate that HBV is not only a weak inducer of innate immunity, but also interferes with pDC function which may contribute to HBV persistence.

MATERIALS AND METHODS

HBV, proteins and TLR ligands

HepG2.215-derived HBV particles were generated and purified as described before [19]. Recombinant CHO-derived HBsAg and recombinant HBeAg or HBcAg derived from E. coli are all from Prospec (Rehovot, Israel). G.M.G.M. Verjans and G.F. Rimmelzwaan (both Dept of Virology, Erasmus MC) provided HSV-1 (MOI 10) and influenza virus (strain H1a, MOI 0.2), respectively. Synthetic TLR ligands used include CpG-2336 (class A 10 mg/ml, Coley Pharma, Düsseldorf, Germany) and Loxoribine (Lox 0.4 mM, Invivogen, San Diego, CA).

Purification and activation of pDC

PBMC and pDC were isolated from peripheral heparinized blood samples or from buffy coats obtained from healthy blood donors using Ficoll density gradient centrifugation. For pDC isolation, Ficoll density gradient centrifugation was followed by CD19+ cell depletion and positive BDCA4+ pDC selection using a PE-conjugated monoclonal anti-BDCA-4 antibody and anti-PE MACS microbeads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) or FACSorting (FACS Aria, Beckton Dickinson, Alphen a/d Rijn, The Netherlands). Isolated pDC were analyzed for purity and viability using anti-BDCA2-FITC (Miltenyi) and 7-AAD (eBioscience, San Diego, USA) by flow cytometry and only used for experiments if more than 95% pure and viable. Where indicated monocytes were either depleted from PBMC or purified with anti-CD14 MACS microbeads and LD-columns or MS-columns (Miltenyi), respectively, according to manufacturer's instructions. Total PBMC ($1x10^6$ cells/ml) or pDC ($2x10^4$ cells/ml) were resuspended in RPMI 1640 (Lonza, Basel, Switzerland) containing 10% heat-inactivated fetal calf serum (FCS, Hyclone, Logan UT), 100 U/ml Penicillin, 100 mg/ml Streptomycin (Breda, The Netherlands) and IL-3 (20 ng/ml, Miltenyi) where indicated.

NK cell activation

PDC were isolated from PBMC by FACsorting and $CD3^-CD56^+$ NK cells were isolated (purity and viability >95%) from PBMC from the same donor with an NK cell isolation kit (Miltenyi Biotec, Germany) according to the manufacturer's instructions. pDC were co-cultured with $1x10^5$ NK cells in a 1:5 ratio in RPMI 1640 containing 10% FCS, penicillin/streptomycin, Hepes and IL-3

either with or without CpG and HBV (200 geq/pDC). After 48h, supernatants were harvested for the detection of IFN-γ production by ELISA (eBioScience) and cells were harvested to examine NK cell activation by flow cytometry.

Flow cytometric analysis surface (bound) molecules

To evaluate the phenotype of pDC, cells were harvested and stained with combinations of anti-BDCA4-PE (Miltenyi), anti-CD80-FITC (MAB104; Immunotech, Marseilles, France), anti-CD86-APC (2331; BD Biosciences), anti-HLA-DR-PerCP (243; BD Biosciences) and anti-CD40-APC (5C3; BD Biosciences) in PBS containing 1% heat-inactivated FCS and 0.02% NaN_3. HBsAg binding was detected with anti-HBsAg-FITC (Acris Antibodies GmbH, Hiddenhausen, Germany). To investigate NK cell activation, cells were stained with a combination of antibodies directed against CD56 (MY31), CD69 (L78) and CD25 (2A3, all BD Bioscience). Cells were analysed by flow cytometry (FACS CantoII or FACScalibur) and FACS Diva or CellQuest Pro software (all Beckton Dickinson, Alphen a/d Rijn, The Netherlands). Corresponding isotype-matched control antibodies were used to determine background staining.

Phospho-S6 and Phospho-IRF7 analysis

Total PBMC ($1x10^6$ cells/250 ml) or purified pDC ($5x10^3$ cells/250 ml) were stimulated in the presence or absence of HBV or viral proteins (5 µg/ml) at 37ºC. After 5 -180 min, cells were fixed with 2 % formaldehyde, washed with PBS/1% FCS/0,02% NaN_3 and incubated with 0.5 % Saponin. Antibodies against Phospho-S6 (pSer235/236, Bioké, Leiden, The Netherlands) and phospho-IRF-7 (pS477/pS479; K47-671, BD Biosciences) followed by goat-anti-rabbit-biotine (Dako, Glostrup, Denmark) were diluted in 0.5% Saponin and added for 15 minutes. Then, cells were stained with streptavidin-PerCP (BD Biosciences) and analyzed by flow cytometry.

Quantitative PCR for IFN-α genes

Isolated pDC were stimulated for 2 or 4 hours with CpG in the absence or presence of HBV. Cells were lysed in Trizol (Sigma-Aldrich) and stored at -80°C until further use. RNA was precipitated with 75% ethanol and isolated using RNeasy mini colums (Qiagen, Venlo, The Netherlands). cDNA was synthesized with iScript cDNA synthesis kit (BioRad laboratories BV) according to manufacturer's instructions. Real-time PCR was performed with the following primer pairs: GAPDH_F 5'-AGG TCG GTG TGA ACG GAT TTG-3' and GAPDH_R 5'TGT AGA CCA TGT AGT TGA GGT CA-3', IFN-α2_F 5'-AAT GGC CTT GAC CTT TGC TT-3' and IFN-α2_R 5'-CAG CTT GAC TTG CAG CTG AG-3', IFN-α8_F 5'-TGG TGC TCA GCT ACA AGT CAT T-3' and IFN-α8_R 5'-TAC CCA GGC TGT GAG TCT GA-3' under standard conditions (annealing temperature 63°C; 40 cycli; MyIQ iCycler, Biorad). Gene expression was calculated using the ΔΔCT method.

Cytokine production by pDC

IFN-α and TNF-α producing pDC were quantified by incubating the cells during the last 3h of 5h cultures with 10 µg/ml Brefeldin A (Sigma-Aldrich, St. Louis, MO). After harvesting, cells were stained with anti-BDCA4-PE (Miltenyi) and anti-CD123-biotin (BD Pharmingen, USA). After washing with PBS/1% FCS/0,02% NaN_3, cells were fixed and permeabilized with Intraprep (Beckman Coulter, Miami, Florida, USA) according to the manufacturer's instructions and stained with anti-IFNα-FITC (Kordia, Leiden, The Netherlands), anti-TNF-α-APC (Caltag-Medsystems, Buckingham, UK) and streptavidin-PerCP (BD Biosciences). The total amount of cytokines produced by purified pDC was determined after 24h of culture. Supernatants were examined for the production of TNF-α (eBioscience), IL-6 (Biosource International, Nivelles, Belgium), IL-8 (Biosource), IP-10 (Invitrogen) and IFN-α (Bender Medsystems, Vienna, Austria) by ELISA.

RESULTS

HBV does not activate pDC

HBV is a DNA virus that replicates via an RNA intermediate. In theory, HBV may thus be able to activate pDC via TLR7 and/or TLR9. To investigate whether HBV is able to activate pDC, PBMC were isolated from healthy controls and cultured in the presence of HBV. As a comparison, PBMC were also stimulated with known synthetic and viral TLR7 and TLR9 ligands including CpG, Lox, HSV-1 and influenza virus. Intracellular IFN-α was investigated in pDC after 5 hr incubation. Influenza virus and HSV-1 and CpG and to a lesser extent Lox induced pDC to produce IFN-α. In contrast, HBV did not give rise to IFN-α producing pDC (Fig. 1). Similar data were observed for TNF-α.

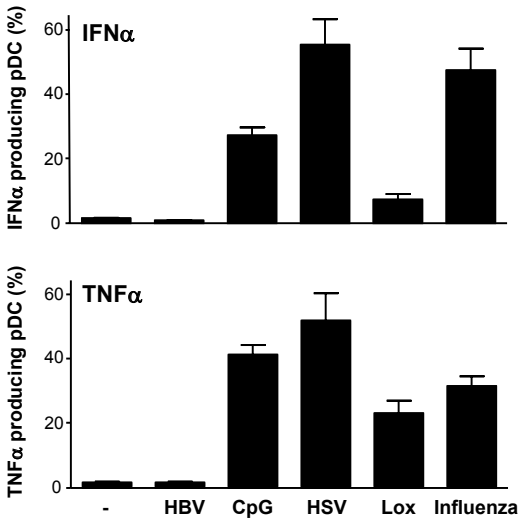

Fig. 1 HBV does not activate pDC.
PBMC were cultured in the presence or absence of HBV (100 geq/cell), CpG, HSV-1, Lox or Influenza. After 5h, cells were harvested and IFNα and TNF-α producing pDC were determined as described in Materials and Methods. Similar data were observed for purified pDC. Data are presented as mean±SEM of 10 independent experiments with different donors.

Though HBV was not able to induce cytokine production by pDC, the virus might be able to induce pDC maturation. Whereas viruses like HSV-1 and influenza as well as the synthetic TLR7 and TLR9 ligands upregulated the expression of CD40, CD80, CD86, and to a minor extent HLA-DR, HBV only marginally increased the expression of HLA-DR without affecting the expression of CD40, CD80 and CD86 (Table 1).

Table 1 Effect of HBV on pDC maturation

	CD40		CD80		CD86		HLA-DR	
	control	HBV	control	HBV	control	HBV	control	HBV
Medium	6.8±1.7	5.0±0.1	23±9	17±6	46±21	35±16	791±385	993±397
HBV	5.0±0.1	ND	17±6	ND	35±16	ND	993±397	ND
CpG	107±15	32±5*	89±25	60±26	122±33	47±20*	911±257	1251±419
HSV	62±21	50±19*	38±13	42±19	72±27	85±42	837±206	1100±425
Lox	44±3	44±5	73±21	89±27	101±21	115±32	1136±226	1368±377
Influenza	154±24	142±19	90±10	89±11	169±25	137±10	780±158	856±174

PBMC were cultured in the presence or absence of HBV (100 geq/cell), CpG, HSV-1, Lox or Influenza. After 24h, cells were harvested and the expression of CD40, CD80, CD86 and HLA-DR on pDC was determined by flow cytometry. Data are presented as mean±SEM fluorescent intensity of 3 independent experiments with different donors. Similar data were observed for purified pDC. ND, not determined; *p<0.05, paired t-test.

HBV directly interacts with pDC resulting in impaired cytokine production

To determine whether HBV mainly behaves as a stealth virus for pDC, or that it does has an active role in the regulation of pDC function, PBMC were activated in the presence or absence of HBV. HBV dose-dependently inhibited the CpG-induced IFN-α response by pDC, which could be already observed with 0.08 geq/cell (Fig. 2A). Similar data were found for CpG-induced TNF-α producing pDC (Fig. 2A).

The reduced pDC function observed in PBMC cultures exposed to HBV does not mean that HBV directly interacts with pDC. Therefore, pDC were purified and exposed to CpG, either in the presence or absence of increasing doses of HBV. Again, HBV significantly reduced the capacity to produce IFN-α as demonstrated by reduced number of IFN-α producing pDC (Fig. 2B) and a reduction in total IFN-α produced (Fig. 2C). Although on a per cell basis more HBV particles seemed to be required for significant immune regulatory effects on purified pDC (Fig. 2C), the number of HBV genome equivalents per pDC present in pure pDC cultures compared to the ±0.2% pDC present in total PBMC cultures was even 10-50 times less.

Nevertheless, the relative inhibition of CpG-induced IFN-α producing pDC was more pronounced when whole PBMC cultures were exposed to HBV. This was not simply due to positive selection on BDCA4 as suggested before [20], since negative selection of pDC revealed similar results (data not shown). Then whole PBMC were compared with PBMC depleted of monocytes, T, B or NK cells, and cultures of purified pDC were compared with purified pDC supplemented with $CD3^+$, $CD14^+$, $CD19^+$, or $CD56^+$ cells. These experiments revealed that especially $CD14^+$ monocytes influenced CpG-induced IFN-α production. PBMC depleted of monocytes showed

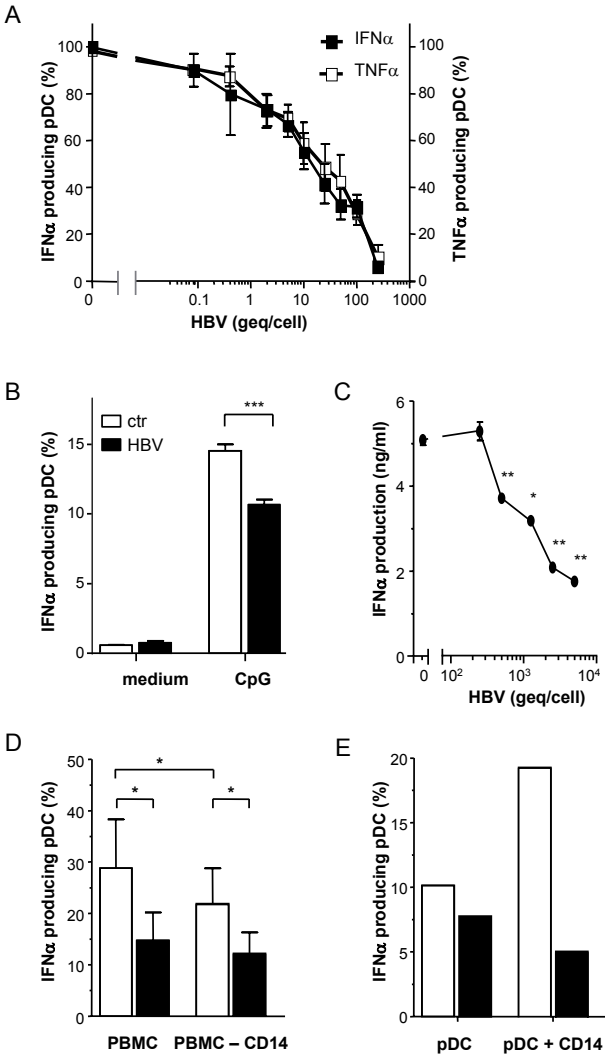

Fig. 2 HBV dose-dependenty inhibits production of IFN-α and TNF-α by pDC.

(A) PBMC were stimulated with CpG in the presence or absence of increasing doses of HBV. After 5h, cells were harvested and the frequency of IFN-α and TNF-α producing pDC were determined. Data are presented as mean±SEM of 6 independent experiments with different donors. (B) Purified pDC were stimulated with CpG in the presence or absence of 2500 geq/cell HBV for 5h. The frequency of IFN-α producing pDC was determined and expressed as mean±SEM of 18 independent experiments with different donors. (C) Purified pDC were cultured with different doses of HBV. After 24h, supernatants were harvested and total IFN-α production was determined by ELISA. Data are presented as mean±SEM of 16 independent experiments with different donors. *p<0.05, **p<0.01, p<0.001, paired t-test. (D) PBMC or PBMC depleted of CD14+ cells were cultured with CpG either in the presence or absence of HBV (100 geq/cell) for 5h. Intracellular IFN-α production by pDC was measured by flow cytometry. Data presented are mean±SEM of 6 independent experiments with different donors. *p<0.05, paired t-test. E: pDC were cultured alone or with purified CD14+ cells in a 1:5 ratio and stimulated with CpG in the presence or absence of HBV (1000 geq/pDC). Intracellular IFN-α production by pDC was measured by flow cytometry. Shown are the percentages IFN-α producing pDC within the BDCA4+CD123+ cells derived from a representative experiment out of 3 independent experiments.

reduced numbers of IFN-α positive pDC (Fig. 2D), whereas the addition of monocytes to purified pDC cultures enhanced the frequency of IFN-α-producing pDC (Fig. 2E). Moreover, this monocyte-mediated IFN-α production by pDC was completely abolished by HBV (Fig. 2E). The other cell populations did not significantly contribute to IFN-α production by pDC (data not shown). These data demonstrate that HBV inhibits pDC function via a direct effect on pDC that can be further enhanced by monocytes.

HBV inhibits CpG induced pDC maturation and function

Whether HBV also interferes with CpG-induced pDC maturation was examined by analysing the expression of co-stimulatory molecules on pDC. CpG resulted in upregulation of CD40, CD80 and CD86, which was significantly inhibited by HBV (Table 1). To assess whether HBV could also interfere with pDC maturation and cytokine production induced by other pDC activating stimuli, purified pDC were activated with HSV-1, Lox and influenza virus either with or without HBV. As described above, HSV, influenza virus and the synthetic TLR ligands all induced pDC maturation albeit to a variable extent. HBV significantly inhibited HSV-induced CD40 expression. Most pronounced inhibitory effects of HBV were observed for CpG-induced pDC maturation as demonstrated by diminished CD40, CD80 and CD86 upregulation (Table 1). With regard to pDC function, HSV-1 and influenza virus induced pDC to produce both IFN-α (Fig. 3A,B) and TNF-α (Fig. 3C), whereas Lox mainly induced TNF-α. Except for CpG, HBV did not affect the cytokine production induced by these stimuli (Fig. 3). Since HSV-1 can trigger TLR9, but also other innate immune receptors, and Lox and influenza are known to trigger TLR7, the inhibitory effect of HBV seemed to be restricted to pure TLR9 triggering.

HBV diminishes IFN-α transcription by inhibiting TLR9-induced phosphorylation of IRF7

It was recently found that TLR9-induced IFN-α production by pDC requires mTOR activation [21] resulting in phosphorylation of IRF7 followed by IRF7 translocation to the nucleus and IFN-α gene transcription. To assess whether HBV interferes with mTOR-induced IRF7 phosphorylation, pDC were stimulated with CpG or Lox or left untreated in the presence or absence of HBV, and analysed by intracellular flow cytometry for the presence of phosphorylated S6, a downstream target of mTOR [22]. As expected, CpG stimulation of pDC induced phosphorylation of S6 (Fig. 4A). HBV significantly reduced the CpG-induced phosphorylation of S6, whereas Lox-induced S6 phosphorylation was hardly affected (Fig. 4B), which is compatible with the lack of inhibitory effects of HBV on Lox-stimulated pDC (Fig. 3). As a consequence, reduced CpG-induced IRF7 phosphorylation (Fig. 4C) and reduced levels of IFN-α2 and IFN-α8 mRNA were detected by quantitative RT-PCR analysis of pDC cultured in the presence of HBV (Fig. 4D).

HBV inhibits cytokine production and NK cell activation by pDC

Although pDC are known as the main producers of IFN-α, they produce more cytokines involved in anti-viral immunity. In addition, pDC are known to promote NK cell activation via pDC/NK cell

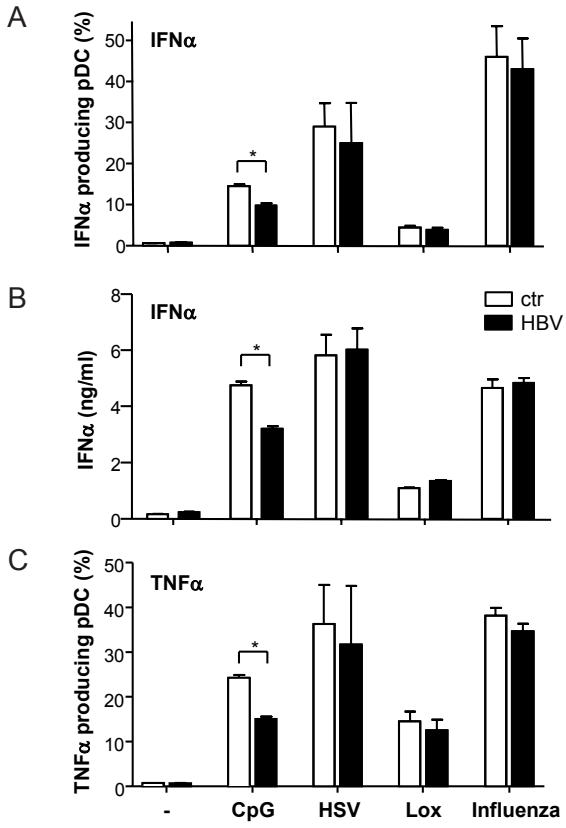

Fig. 3 HBV inhibits CpG-induced pDC function.

Purified pDC were cultured in medium or CpG, HSV-1, Lox or Influenza virus in the presence or absence of HBV (2500 geq/cell) for 5h (A,C) or 24h (B). IFN-α production was determined by intracellular flow cytometry (A) and ELISA (B), and TNF-α production was determined by flow cytometry (C). Data are expressed as mean±SEM of 3-10 independent experiments with different donors. *p<0.05, paired t-test.

reciprocal interaction [11]. According to previous studies, stimulation of purified pDC by CpG resulted in the induction of TNF-α, IP-10 and IL-6 (Fig. 5A-C), whereas CpG did not affect the production of IL-8 (Fig. 5D). HBV significantly inhibited the CpG-induced production of TNF-α, as was already demonstrated by intracellular flow cytometry. CpG-induced production of IP-10 and IL-6 were also inhibited, whereas the production of IL-8 was not significantly affected (Fig. 5). Addition of pDC to purified NK cells resulted in strong NK cell activation within 48h as demonstrated by the upregulation of CD69, CD25 and the induction of IFN-γ production (Fig. 5E,F; data not shown). NK cell activation was even further enhanced when pDC were stimulated with CpG as shown by the enhanced CD25 expression and increased IFN-γ production compared to non-stimulated cultures (Fig. 5E,F). Although HBV neither affected the pDC-induced upregulation of CD69 nor CD25 on NK cells (Fig. 5E; data not shown), NK cell-derived IFN-γ production induced by CpG-activated pDC was significantly decreased (Fig. 5F).

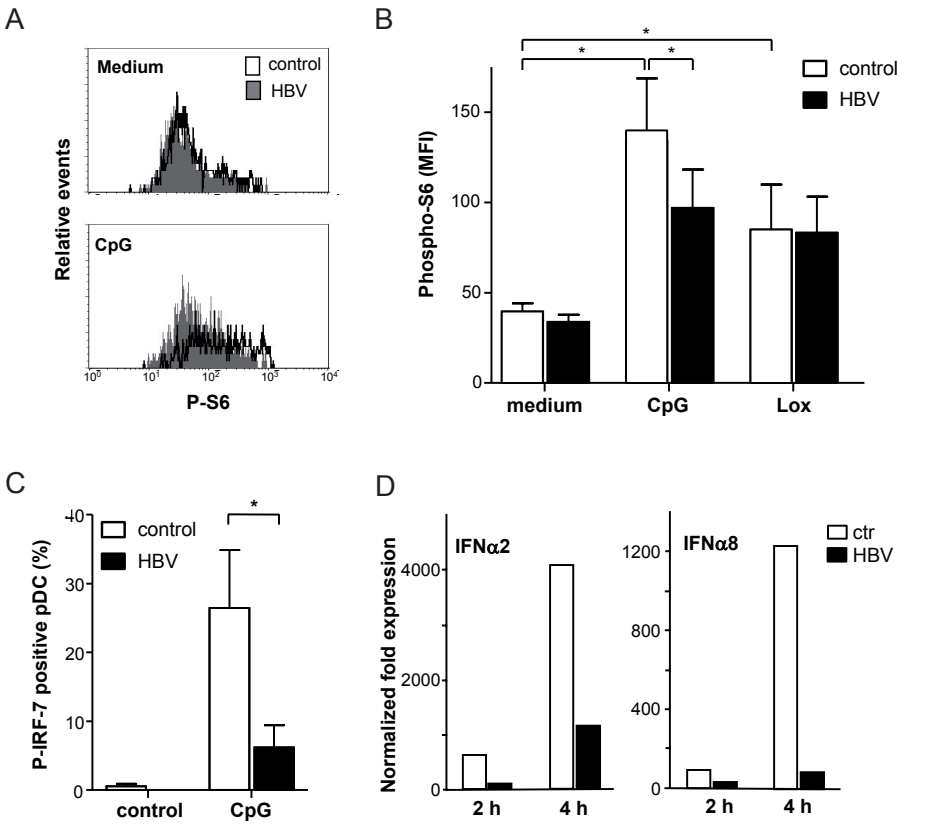

Fig. 4 HBV inhibits S6 phosphorylation and IFN-α gene transcription.
(A) PBMC were cultured with CpG in the presence or absence of HBV (100 geq/cell). Cells were harvested and analysed for intracellular expression of phosphorylated S6 by flow cytometry. Data show the expression of phosphorylated S6 in BDCA4+CD123+ pDC and is representative for 8 independent experiments with different donors. Similar data were observed for purified pDC. (B) PBMC were cultured with or without CpG or Lox in the presence or absence of HBV (100 geq/cell). Cells were harvested and analysed for intracellular expression of phosphorylated S6 by flow cytometry. Data show the mean±SEM expression of phosphorylated S6 in BDCA4+CD123+ pDC in MFI from 6 independent experiments with different donors. *p<0.05, Wilcoxon signed rank test. (C) PBMC were cultured with CpG in the presence or absence of HBV (100 geq/cell) for 3h. Cells were harvested and analysed for intracellular expression of phosphorylated IRF7 by flow cytometry. Data present the mean±SEM percentage of cells positive for phosphorylated IRF7 in BDCA4+CD123+ pDC from 5 independent experiments with different donors. Similar data were observed with purified pDC. (D) Purified pDC were cultured with CpG in the presence or absence of HBV (2000 geq/cell) for 2 or 4h. Cells were harvested and IFN-α2 and IFN-α8 mRNA were determined as described in Material and Methods. Data are representative for 3 independent experiments with different donors.

HBsAg binds to pDC and suppresses their function

To unravel if the whole virus is required for HBV-induced modulation of pDC function or that also the single HBV proteins influence pDC function, HBsAg, HBcAg, and HBeAg were investigated for their effect on CpG-induced IFN-α production. Dose response studies revealed that HBeAg and especially HBsAg, but not HBcAg dose-dependently reduced CpG-induced IFN-α production by purified pDC (Fig. 6A). In line, only HBsAg significantly inhibited CpG-induced

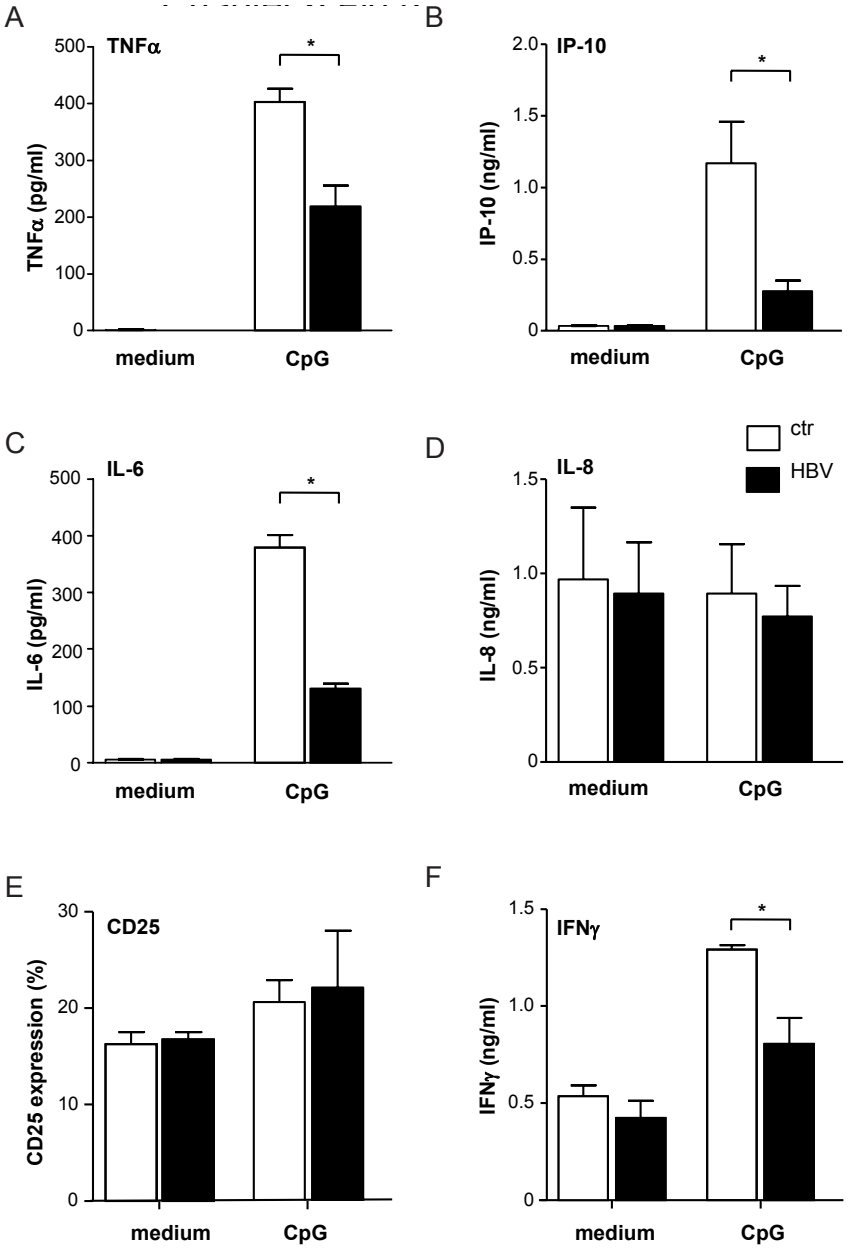

Fig. 5 HBV inhibits cytokine production and pDC-induced NK cell activation.

(A, B, C, D) Purified pDC were cultured with CpG in the presence or absence HBV (2000 geq/cell) for 24h. Supernatants were analysed for the presence of TNF-α (A), IP-10 (B), IL-6 (C) and IL-8 (D) by ELISA. Data demonstrate mean±SEM of 8 independent experiments with different donors. (E,F) PDC were cultured with NK cells either in the presence of CpG and/or HBV as described in Materials and Methods. After 48h, NK cell activation was determined by analyzing CD25 expression on CD56+ cells by flow cytometry (E) and NK cell function was determined by analyzing IFN-γ in culture supernatants by ELISA (F). Data show the mean±SEM of 7 independent experiments with different donors. *p<0.05, Wilcoxon signed rank test.

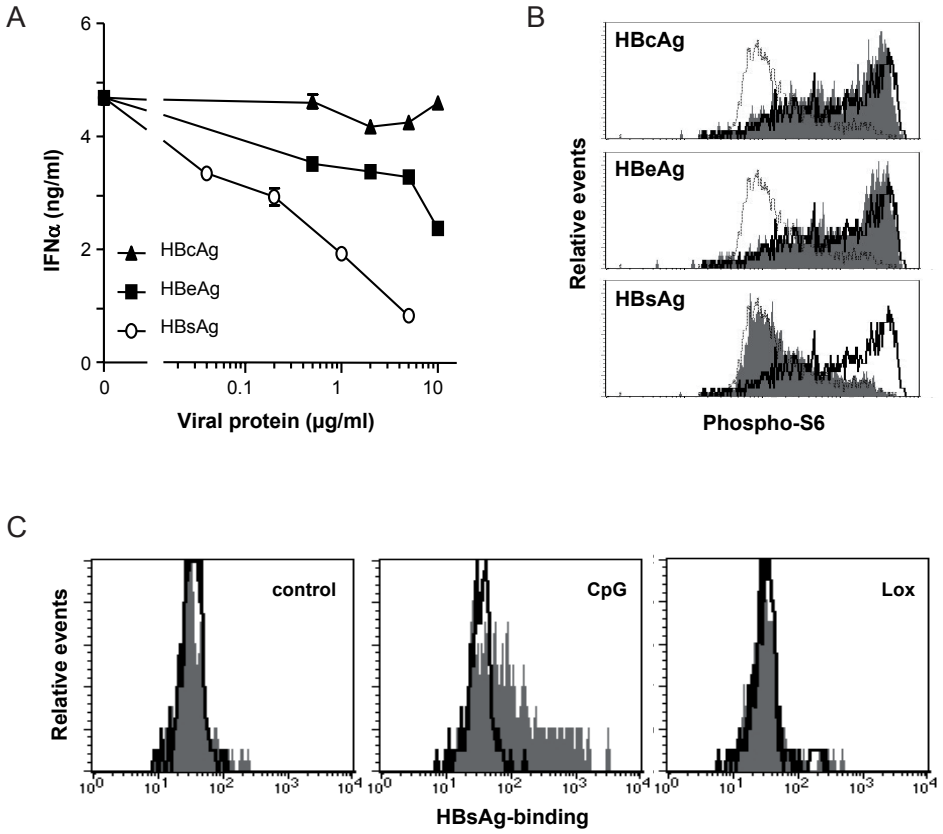

Fig. 6 Especially HBsAg inhibits pDC function.
(A) Purified pDC were cultured with CpG in the presence or absence of increasing doses of HBcAg, HBeAg or HBsAg for 24h. Supernatants were harvested and analysed for IFN-α by ELISA. Data presented are mean±SEM of at least 8 independent experiments with different donors. (B) PDC were cultured with or without CpG in the presence or absence of 5 µg/ml HBcAg, HBeAg, or HBsAg. Cells were harvested and analysed for intracellular expression of phosphorylated S6 by flow cytometry. Data are representative for 3 independent experiments with different donors. Open/thin histogram: non-stimulated pDC; open/thick: pDC+CpG; Filled: pDC+CpG+viral protein. (C) PDC were cultured with or without Lox or CpG and with or without HBsAg. After 4h, cells were harvested and surface bound HBsAg was detected by flow cytometry.

phosphorylations of S6 (Fig. 6B) and IRF-7 (data not shown). Like HBV, HBsAg did not inhibit Lox-induced cytokine production by pDC (data not shown).

Since HBsAg and HBV display similar immune regulatory effects on pDC with regard to TLR7- and TLR9-induced functional alterations and intracellular signalling, the interaction between HBsAg and pDC was studied in more detail. The lack of major interference with Lox-induced S6 phosphorylation is in line with the limited effects of HBV/HBsAg on Lox-mediated pDC function compared to CpG and suggests that HBV/HBsAg especially interacts with pDC upon TLR9 stimulation. Therefore, cells were cultured under standard conditions with or without CpG or Lox either in the presence or absence of HBsAg for 4h. In addition, cells were stimulated with CpG and Lox for 2h at 37°C, then cells put on ice and HBsAg was added. Subsequently, pDC

surface binding of HBsAg was determined. In both types of experiments, a very low binding of HBsAg was observed in cultures with medium alone or Lox, whereas pDC stimulated with CpG strongly bound the HBV envelope protein (Fig. 6C). The preferential binding of HBsAg to CpG-stimulated pDC explains the ability of the virus to interfere especially with CpG-induced pDC function. Similar to the effect of the whole virus, HBsAg inhibited CpG-induced TNF-α (Fig. 7A), IP-10 (Fig. 7B) and IL-6 (Fig. 7C) production without significantly altering the production of IL-8 (Fig. 7D). HBeAg also reduced TNF-α production, but did not significantly influence the secretion of the other cytokines. HBcAg did not modulate pDC function (Fig. 7). Of note, neither HBV nor its viral proteins reduced pDC viability as determined by flow cytometric analysis of intracellular active caspase-3 as well as the binding of Annexin-V and 7AAD (data not shown).

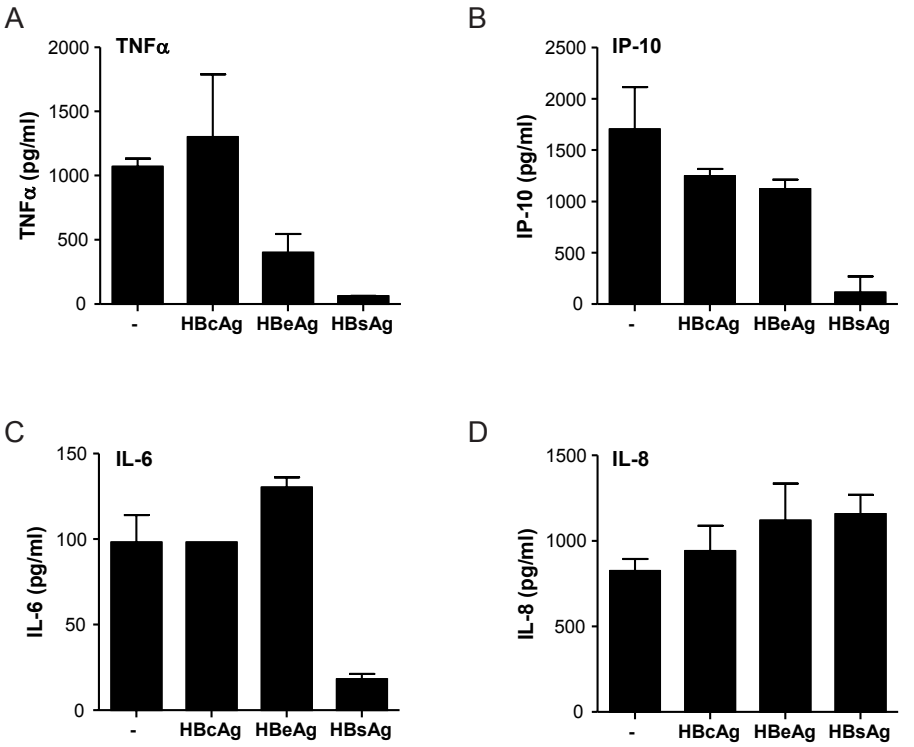

Fig. 7 HBeAg and especially HBsAg inhibit cytokine production by pDC.
Purified pDC were cultured with CpG in the presence or absence of 5 μg/ml HBcAg, HBeAg or HBsAg for 24h. Supernatants were harvested and analysed for TNF-α (A), IP-10 (B), IL-6 (C), and IL-8 (D) by ELISA. Data presented are mean±SD of duplicates representative for at least 6 independent experiments with different donors.

DISCUSSION

PDC play a central role in anti-viral immunity due to their rapid and profound release of type I IFN upon viral recognition. An initial type I IFN response directly after infection with HBV is seemingly lacking and patients chronically infected with HBV show functional deficits in pDC with regard to IFN-α production [4,5,12]. The present study demonstrates that HBV does not activate pDC as assessed by cytokine production and upregulation of co-stimulatory molecules. Moreover, HBV actively interfered with TLR9-induced pDC function, resulting in a dose-dependent inhibition of cytokine production and pDC maturation. HBsAg, and to a limited extent HBeAg, which were used in concentrations found in hepatitis B patients' circulation [18], showed similar immune regulatory effects as HBV thereby demonstrating that the immune regulatory effects of HBV do not require active infection.

PDC respond to viruses primarily through the recognition of pathogen-associated molecular patterns including TLR9, which recognizes viral DNA [8,9]. Two factors seem to be key for the induction of large quantities of type I IFN in pDC: (a) the ability of the TLR ligand to bind its receptor in the early endosomal compartments [23,24] and (b) the phosphorylation and nuclear translocation of IRF7 [25]. HBV DNA is present in peripheral blood pDC isolations [17,26], but no evidence exists for HBV infection and viral replication in pDC. The lack of IFN-α production upon exposure to HBV could be due to the fact that HBV is not taken up by pDC and is therefore not able to reach TLR9. Here we showed that, although limited, HBsAg is able to bind to non-stimulated pDC. However, whether pDC are able to internalize HBV particles remains to be elucidated. Another explanation for the absent IFN-α induction is the active suppression of IFN-α production as was observed for CpG-induced type I IFN. The exposure to HBV not only lead to decreased pDC function, but also resulted in diminished pDC-induced NK cell function. The impaired crosstalk between different immune cells may partially explain the failing induction of effective anti-viral immunity. Whether the impaired DC-NK cell crosstalk could explain the defective IFN-γ production by NK cells circulating in patients with chronic hepatitis B [27] remains unknown. It was recently found that TLR9-induced IFN-α production by pDC requires mTOR activation [21]. How exactly mTOR regulates the production of type I IFN is not known, but one of the models proposed is mTOR-induced phosphorylation of S6K that promotes formation of the TLR9-MyD88 complex. As observed for HBV, pharmacological inhibition of mTOR by rapamycin reduced the production of CpG-induced IFN-α, TNF-α and IL-6 (data not shown) [21]. Here, we demonstrated that HBV reduced the phosphorylation of S6, which was paralleled by reduced IRF7 phosphorylation and diminished IFN-α gene transcription. The inhibitory effect of HBsAg was completely overlapping with HBV, as demonstrated by inhibition of S6 and IRF7 phosphorylation and production of IFN-α, TNF-α, IL-6 and IP-10, and was much stronger than the regulatory effect of HBeAg. At what level HBV and HBsAg interfere with mTOR-induced S6 phosphorylation remains to be elucidated.

The differential effects of HBsAg and HBeAg with regard to intracellular signalling and cytokine production, could be explained by the use of different receptors. Several putative binding factors have been described for HBsAg, such as human serum albumin, asialoglycoprotein receptor, and heparin, but their exact role in HBV attachment remains unclear [28]. Since HBV surface antigens are glycoproteins, the involvement of a C-type lectin receptor at the DC surface in viral recognition and blockade of TLR9-induced IFN-α production seems plausible. Crosslinking of the pDC-specific C-type lectin DCIR resulted in a specific TLR9, but not TLR7-mediated inhibition of TNF-α and IFN-α [29], which is compatible to the immune modulatory effects observed for HBV. Simultaneous reduction in IFN-α, TNF-α and IL-6 production, as shown here for HBV, has also been reported for crosslinking the pDC-specific C-type lectin ILT7-FcεRIγ [30]. Several other viruses have been shown to block TLR9-induced pDC activation and function, but not TLR7-mediated activation, including HIV [31,32] and HCV [33,34], which may crosslink similar cell surface receptors as HBV. Whether also these viruses preferentially bind to TLR9-activated pDC compared to TLR7-activated pDC, as we found for HBV is not documented.

Concerning HBV, the increased binding of HBsAg to CpG-stimulated pDC suggests the involvement of a factor, either membrane bound or soluble, that is strongly induced by TLR9 triggering. The binding of HBV to BDCA-2 [35,36] to explain the inhibitory effect of HBV on pDC function seems therefore not very likely. BDCA-2 is highly expressed on non-stimulated pDC and is downregulated upon stimulation with CpG (data not shown). Furthermore, BDCA-2 crosslinking is known to inhibit HSV and CpG-induced pDC function to a similar extent and in our hands crosslinking of BDCA-2 did not inhibit S6 phosphorylation (data not shown).

The finding that HBV DNA is present in peripheral blood pDC isolates of a subgroup of chronic HBV patients [13,17] and the impaired pDC function in chronic HBV patients reported by several independent investigators, but challenged by others (reviewed in Woltman *et al.* [12]), might be explained by the molecular interaction between HBV/HBsAg and/or HBeAg and pDC combined with patient diversity. Host factors and disease state may influence the rate of interaction between HBV virions and/or subviral particles and pDC that could range between a rather weak interaction in case of non-stimulated pDC and a strong interaction with pDC under specific conditions.

The immune regulatory effect of both virions and viral proteins may partially explain the impaired cytokine production by pDC derived from patients chronically infected with HBV [12]. Not HBV DNA reduction alone, but especially loss of serum HBeAg during successful treatment of patients with potent anti-viral drugs was suggested to be responsible for partial restoration of circulating pDC numbers and the impaired IFN-α production by pDC [14,26]. These findings could be ascribed to the possible immune regulatory effect of HBeAg, as has been suggested before [37]. Furthermore, although HBV DNA levels decreased upon treatment with these anti-viral drugs, serum HBsAg levels were not reported and could have been of influence on pDC function, as demonstrated in the present study.

The supportive effect of monocytes in regulating pDC function upon interaction with HBV has also been reported for HCV [38]. In contrast to HCV, we could not detect HBV-induced TNF-α or IL-10 in monocyte cultures and HBV-induced regulatory effects could not be overcome by IL-10 neutralization (data not shown). Interaction between HBV/HBsAg and monocytes have been reported [39], but so far the mechanism underlying monocyte-mediated regulation of pDC function is not known.

Since a considerable number of patients chronically infected with HBV do not demonstrate an adequate response towards the virus upon treatment with the standard immune modulatory therapy, i.e. pegylated-IFN-α [40], novel anti-viral strategies are needed. For the treatment of chronic HCV, promising results were obtained with the TLR9 agonist CpG10101 [41]. The finding that HBV strongly interacts with CpG-activated cells may disrupt the anti-viral effects of such immune stimulating agents. Therefore, the data presented in the current study could be very useful for the design of novel immune modulatory strategies for the treatment of chronic HBV.

In conclusion, HBV does not activate, but actively interferes with S6K-induced pDC function. The data presented here provide new insight into the mechanism by which HBV is able to evade anti-viral immunity and may aid in the development of effective immunomodulatory therapies to clear the viral infection.

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CHAPTER 7

Interferon-α treatment differentially affects TLR7 and TLR9-induced plasmacytoid dendritic cell function of chronic Hepatitis B patients

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ABSTRACT

Toll-like receptor (TLR) triggering of plasmacytoid dendritic cells (pDC) is a key step in defense against viral infections, leading to the mass production of the anti-viral cytokine interferon-α (IFN-α). However, acute infection with Hepatitis B virus (HBV) fails to induce a strong anti-viral IFN-α response and pDC of chronic HBV patients have been shown to produce less IFN-α compared to healthy controls. To investigate whether PEG-IFN therapy could restore the impaired pDC activation in chronic HBV patients, the effect of PEG-IFN treatment on TLR7/9-induced pDC function was examined *in vitro* and *in vivo*.

Before start of PEG-IFN therapy, *in vitro* presence of IFN-α in pDC cultures increased the TLR9- and TLR7-induced IFN-α production, which could be blocked by neutralization of the IFN-α receptor. PEG-IFN therapy augmented TLR7 mRNA levels in pDC leading to increased IFN-α response after TLR7 triggering, while TLR9 mRNA levels were 2-fold reduced which diminished response to TLR9 triggering. Additionally, pDC became insensitive to the presence of PEG-IFN during culture. Both effects on TLR7/9 triggering and IFN-α sensitivity reversed to baseline levels at 24 weeks post PEG-IFN treatment. Similar data were obtained with isolated pDC, indicating that PEG-IFN therapy directly influences pDC function. No difference between reponders and non-responders to PEG-IFN-α therapy was observed. Thus, response to TLR7 and TLR9 specific ligands is differentially regulated during PEG-IFN therapy by a direct effect on TLR7 and TLR9 expression levels. This effect is directly caused by PEG-IFN therapy and could not be attributed to change in serum HBV load.

INTRODUCTION

Worldwide, more than 400 million people are chronically infected with Hepatitis B virus (HBV) [1]. Although risk factors such as age and route of infection for developing chronic HBV infection after acute exposure have been described, the exact mechanism by which HBV escapes immunity is still not known [2,3]. Characteristic of patients with chronic HBV infections is the ineffective anti-viral immune response towards the virus [4].

Plasmacytoid dendritic cells (pDC) are key players in anti-viral defense, due to their capability of mass production of the anti-viral cytokine interferon-α (IFN-α) upon viral infection [5,6]. Detection of viral genetic material and/or viral replication intermediates occurs trough intracellular immune receptors, including Toll-like receptors (TLR). These TLR reside in the endosomal compartments, where single-stranded RNA triggers TLR7 and double-stranded DNA containing CpG motifs triggers TLR9 [7]. Both TLR7 and TLR9 triggering of pDC lead to the secretion of excessive amounts of IFN-α and also other pro-inflammatory cytokines [8,9]. Next to a strong direct anti-viral effect, IFN-α activates macrophages, natural killer cells and T lymphocytes allowing further priming of anti-viral immunity [5].

A hampered IFN-α response during HBV infection could contribute to development of a chronic infection. In contrast to acute infection with other viruses such as Hepatitis C and Human Immunodeficiency Virus, chimpanzees which are acutely infected with HBV completely lack an initial type I IFN (IFN-α/β) response [10,11]. A recent study showed that type I IFN response in a rare cohort of acute HBV patients is also lacking [12]. In addition, several studies showed impaired pDC function from chronic HBV patients after stimulation with SAC [13], HSV [14], or CpG [15-17] as compared to healthy controls. This defective IFN-α production could be related to the observed reduction in TLR9 expression levels in pDC of chronic HBV patients [17]. Serum HBV load reduction by anti-viral therapy resulted in restoration of pDC numbers and function to near normal levels in some, but not all, patients [14]. Another study showed a slight increase in circulating pDC numbers, without improvement of pDC function upon therapy-induced viral load reduction [18]. This lack of pDC immune response restitution might be a clue why current anti-viral replication therapies do not lead to sustained viral eradication. The compromised IFN-α response of chronic HBV patients might explain the initial susceptibility to PEG-IFN therapy. Since PEG-IFN is an immune modelatory therapy, it might enhance the impaired pDC function of chronic HBV patients. Unfortunately, side effects such as flu-like symptoms, cytopenia and psychiatric adverse events are frequently observed during PEG-IFN therapy [19]. Combined with the fact that sustained response after treatment discontinuation is only achieved in approximately 30% of patients, it would be beneficial if response or non-response to therapy could be predicted at an early stage [19]. Here we investigated the effect of PEG-IFN therapy on pDC phenotype and function, *in vitro* and *in vivo*. Both unfractionated PBMC and purified pDC were used to distinguish between direct and indirect effects on pDC function. Furthermore, we investigated whether the PEG-IFN therapy-induced change in pDC function could predict the clinical response to therapy.

MATERIALS AND METHODS

Patients

Patient characteristics of 21 chronic HBV patients who underwent therapy with pegylated interferon-alpha 2a (PEG-IFN; 100 µg weekly) are given in Table 1. Serum HBV load and alanine aminotransferase levels (ALT) were determined at indicated time points as part of the patient's diagnostic evaluation. Heparinized peripheral blood samples were obtained at baseline and after 12, 24, 36, 48 weeks of therapy from patient 1-12 and after 4, 12 and 48 weeks from patients 13-21. A follow up sample was obtained 24 weeks after treatment, from patients that were not receiving anti-viral therapy at that moment. Patients 1-9, who were recruited from the multicenter PARC study, were enrolled in the Erasmus MC and received PEG-IFN monotherapy. All participants gave informed consent before inclusion.

Table 1 Patient characteristics

Patient #	Age (yr)	Sex	HBV Genotype	Viral load (geq/ml)	ALT (U/l)	HBeAg	Response to therapy
1	34	m	D	8,95E+04	76	E-	Marked
2	31	m	D	9,53E+06	443	E-	Marked
3	52	m	A	4,53E+05	63	E-	Marked
4	36	m	D	3,55E+07	99	E-	Poor
5	30	f	E	3,78E+07	109	E-	Poor
6	47	m	D	7,27E+03	183	E-	Poor[A]
7	35	f	D	4,28E+04	47	E-	Marked
8	35	m	D	1,84E+06	61	E-	Marked
9	34	m	D	8,88E+07	427	E-	Marked
10	42	m	A	1,03E+10	96	E+	Poor[B]
11	37	m	A	2,10E+09	92	E+	Poor
12	41	m	A	2,40E+09	113	E+	Sustained
13	42	m	A/G	1,12E+10	196	E+	Marked
14	27	m	C	1,87E+07	186	E-	Poor
15	19	m	C	3,43E+04	32	E+	Marked
16	32	m	D	5,38E+08	123	E-	Marked
17	58	m	A	6,50E+06	88	E-	Poor[C]
18	49	m	A	1,21E+06	38	E-	Marked
19	43	m	D	5,02E+08	136	E-	Marked
20	57	m	D	6,53E+07	129	E-	Marked
21	17	f	C	7,42E+08	106	E+	Poor

Poor: <3log10 HBV copies/ml decline in viral load; Marked: >3log10 HBV copies /ml decline in viral load; Sustained: <3log10 HBV copies/ml at EOF

[A] Switched to adefovir after 12 weeks

[B] Switched to adefovir after 48 weeks

[C] Switched to entecavir after 12 weeks

Isolation of pDC from peripheral blood of chronic HBV patients

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples using Ficoll-Isopaque gradient centrifugation. PDC ($BDCA4^+$) were isolated from PBMC by negative depletion with anti-CD19-conjugated microbeads, followed by positive selection using anti-BDCA4-PE and anti-PE-conjugated microbeads using the mini-MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity and viability of the isolated pDC were determined by flow cytometry (FACScalibur, Becton, Dickinson and Company (BD), Franklin Lakes, NJ) by staining for BDCA4, BDCA2 and 7AAD (eBioscience, San Diego, CA). Isolated pDC were cultured at 37°C in DC medium consisting of RPMI 1640 (Bio Whittaker, Verviers, Belgium) containing 10% FCS (Hyclone, Logan, UT), penicillin (50 IU/ml) and streptomycin (50 µg/ml; Invitrogen Ltd, Paisley, UK).

Effect of IFN-α on pDC phenotype

Isolated pDC (≥1x10E4 pDC/200 µl) were cultured in round bottom 96-wells plates for 22 or 44 hr with IL-3 (10 ng/ml; Strathmann Gmbh & Co, Hamburg, Germany) in the presence or absence of 40U/ml IFN-α (Schering-Plough Corporation, Kenilworth, NJ). PBMC (1x10E6/500µl) were cultured under similar conditions in flat bottom 24-wells plates. Cells were stained for anti-BDCA4 (Miltenyi), anti-CD123 (BD), anti-CD40 (BD), anti-CD80 (Beckman Coulter, Brea, CA), anti-CD86 (BD), and anti-HLA-DR (BD) and expression levels were determined by flow cytometry. Total and absolute pDC numbers were determined by white blood cell count and flow cytometry after whole blood staining with fluorescein-conjugated anti-CD45 (Beckman), anti-BDCA4, anti-CD11c (BD) antibodies.

IFN-α production by pDC

Isolated pDC (≥5x10E3 pDC/200 µl) or PBMC (1x10E6/200µl) were cultured in round bottom 96-wells plates. Cells were stimulated with LPS (negative control; 10 ng/ml) (Sigma- Aldrich, St. Louis, MO), CpG-A, CpG-B or CpG-C (TLR9; 10 µg/ml; Coley Pharmaceutical, Ottawa, Canada); loxoribin (Lox; TLR7; 400 µM; Invivogen, San Diego, CA); HSV-1 (moi 10; kind gift of Dr. G.M.G.M. Verjans); influenza virus (moi 0,2; kind gift of Dr. G.F. Rimmelzwaan) with or without 40U/ml IFN-α. During the last 3 hr of culture brefeldin (10 µg/ml; Sigma-Aldrich) was present. Timecourse experiments with stimulation times varying from 3 to 24 hr indicated the highest IFN-α production after 5 hr, therefore cells were stimulated for 5 hr in all IFN-α production assays. Intracellular IFN-α and TNF-α production was determined by flow cytometry, after blocking with PBS/0,1% BSA/0.5% humane serum, followed by (intra)cellular staining (Intraprep; Beckman Coulter) with anti-BDCA4-PE, anti-CD123-biotine, anti-streptavidine-PerCP (BD), anti-IFN-α-FITC (PBL Interferon Source, Piscataway, NJ) and anti-TNF-α-APC (Invitrogen). Neutralization assays were performed with anti-type I IFN receptor (IFNAR; 10 µg/ml; PBL Interferon Source) or isotype control antibodies (10 µg/ml).

RNA isolation and realtime PCR

PDC were lysed in Trizol (Sigma-Aldrich) and stored at -80°C until further use. RNA was precipitated with 75% ethanol and isolated using Micro RNAeasy silica columns (Qiagen, Venlo, The Netherlands). cDNA was prepared using AMV reverse transcriptase according to the manufacturer's protocol (Promega, Madison, WI). Real-time PCR was performed for 40 cycles (LightCycler, Roche Applied Science, Indianapolis, IN) with primers specific for TLR7 (F: 5'- CCAGTGTCTAAAGAACCTGGA -3'; R: 5' GGGACAGTGGTCAGTTGGTT -3'), TLR9 (F: 5'- CTGCCCAAATCCCTCATATC -3'; R: 5'- TGCAGAGTC-TAGCATCAGG -3') combined with a FAM-labelled probe (probe 30; Universal ProbeLibrary, Roche Applied Science) and reference gene Abl (F: 5'- TGGAGATAACACTCTAAGCATAACTAAAGGT -3'; R: 5' GATGTAGTTGCTTGGGACCCA -3') combined with a FAM-labelled probe (Applied Biosystems, Foster City, CA). Gene expression was calculated using the ΔΔCT method [20].

RESULTS

IFN-α treatment slightly affects pDC phenotype *in vitro*

Short-term (24 hr) culture of isolated pDC of untreated chronic HBV patients in IL-3 alone induced pDC maturation, as shown by upregulation of HLA-DR and co-stimulatory molecules CD40, CD80 and CD86 (Fig. 1). Exposure to IFN-α slightly interfered with pDC maturation by inhibiting the upregulation of HLA-DR, CD80 and CD86, whereas the expression of CD40 was slightly enhanced. Prolonged (44 hr) exposure to IFN-α showed similar effects on the expression of HLA-DR and CD40, but CD80 and CD86 expression ceased to differ from cells cultured in the absence of IFN-α. Similar results were obtained for pDC in cultures of unfractionated PBMC (data not shown).

IFN-α treatment improves pDC function *in vitro*

Using isolated pDC of untreated chronic HBV patients, TLR7 triggering by Lox and TLR9 triggering by CpG-A resulted in 7±2% (mean±SEM) and 21±5% IFN-α positive pDC, respectively (Fig. 2A). Exposure to IFN-α during culture 2-fold increased the TLR7- and TLR9-induced IFN-α production. TLR7 and TLR9 triggering induced equal amounts of TNF-α producing pDC, which also increased in presence of IFN-α.

Stimulation of PBMC led to higher levels of IFN-α and TNF-α producing pDC, compared to isolated pDC (Fig. 2A). TLR9 triggering was studied in more detail by comparing responses to CpG-A, CpG-B and CpG-C. As expected, very little IFN-α was produced upon CpG-B stimulation, while CpG-C induced an intermediate number of IFN-α producing pDC. The presence of IFN-α significantly increased the number of IFN-α and TNF-α producing pDC with all stimuli. Neutralization of the IFN-α receptor on the cell surface of isolated pDC abolished the stimulatory effect of exposure to IFN-α during TLR9 triggering (Fig. 2B). IFN-α receptor neutralization lead to even less IFN-α production after CpG stimulation compared to control, suggesting that the IFN-α positive feedback loop is involved in CpG-A-induced IFN-α production.

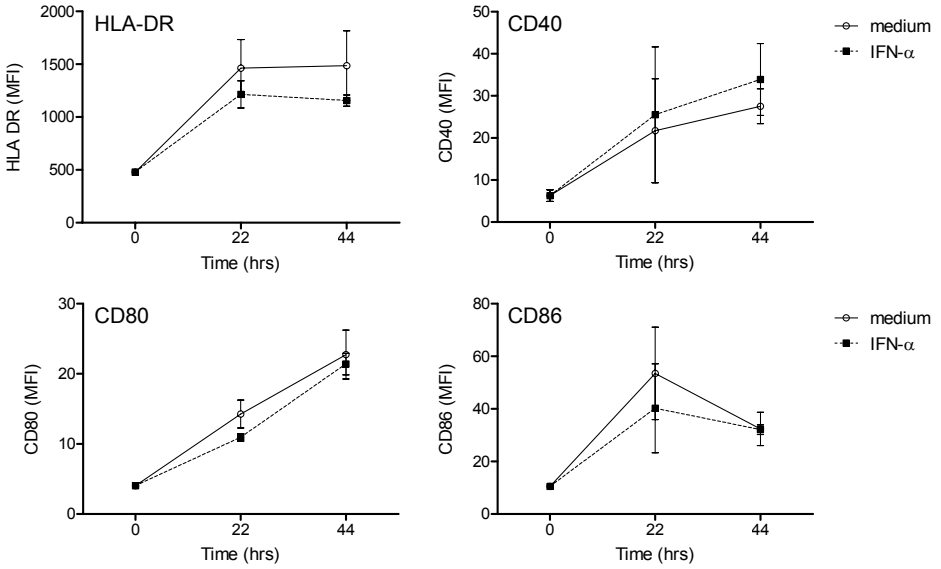

Fig. 1 IFN-α affects pDC phenotype *in vitro*.
Purified pDC obtained from blood of chronic HBV patients were cultured for 22 or 44 hr with IL-3 in the presence or absence of IFN-α. Surface expression of HLA-DR, CD40, CD80 and CD86 on CD123+BDCA4+ pDC was measured by flow cytometry directly after isolation and after culture. Data represent mean±SD of three independent experiments with pDC of different donors.

Clinical outcome of PEG-IFN treatment of chronic HBV patients

To investigate whether the stimulatory effect of IFN-α with respect to the cytokine production could be found *in vivo* and whether these IFN-induced pDC alterations *in vivo* could be linked to clinical response to treatment, pDC phenotype and function were examined of chronic patients during PEG-IFN therapy. PEG-IFN therapy induced a more than 3log decline in serum HBV load in 13 out of 21 patients (62% responders). 24 weeks after treatment discontinuation serum HBV load remained below 1000 copies per ml in two of these 13 responders (Fig. 3A). One of these patients showed loss of serum HBsAg during therapy and is considered a sustained responder. In the other eight out of 21 patients no or only a poor decline in viral load was observed (38% non-responders). Three of these patients converted to anti-viral therapy with nucleotide reverse transcriptase inhibitors and were excluded from this study upon switch of therapy. ALT levels normalized during PEG-IFN therapy in both responder and non-responder groups. Five patients showed a raise in serum ALT levels off-treatment (Fig. 3B).

IFN-α therapy affects numbers, phenotype and function of pDC in blood of chronic HBV patients

Chronic HBV patients receiving PEG-IFN therapy showed a marked reduction in circulating pDC numbers in peripheral blood, independent of the clinical outcome of therapy. In a combined analyses of responders and non-responders, circulating pDC numbers declined from 6405±1172 (mean±SEM) pDC/ml at baseline to 2740±635 pDC/ml after 12 weeks of treatment

(p=0.012). Similarly, the percentage of pDC in the CD45+ cell fraction decreased during PEG-IFN treatment in both responder and non-responder groups (Fig. 4A). After 24 weeks post PEG-IFN treatment, pDC numbers and percentages reversed to baseline levels.

Phenotypic analysis indicated that PEG-IFN therapy induced pDC activation, as shown by a significant increase in CD86 expression in the responder group during the first 12 weeks of

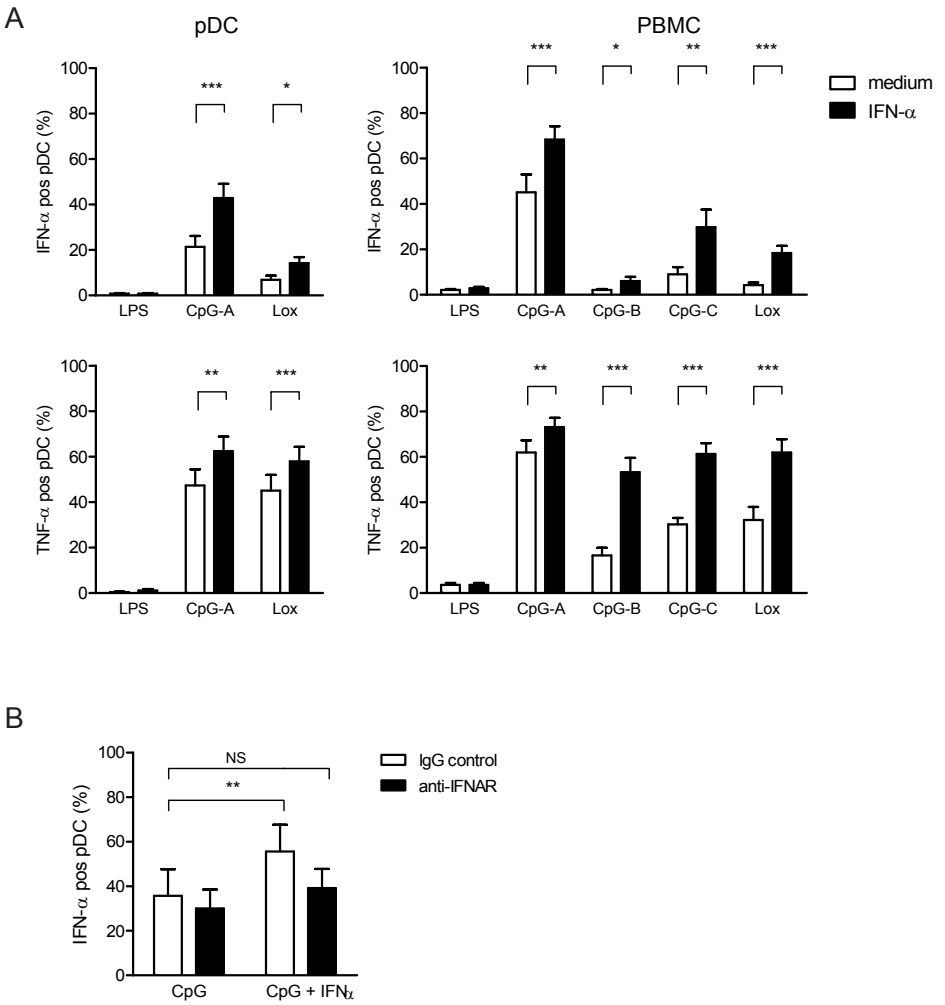

Fig. 2 *In vitro* exposure to IFN-α increased TLR7- and TLR9-induced cytokine production of pDC.
(A) Cells were stimulated for 5 hr with LPS, CpG-A, CpG-B or CpG-C or Lox with or without IFN-α. Intracellular IFN-α and TNF-α were determined by flow cytometric analysis of CD123+BDCA4+ pDC present in either purified pDC (N=8) or unfractionated PBMC (N=9). (B) PBMC were CpG-stimulated with or without IFN-α in the presence of an IgG control or a type I IFN receptor (IFNAR) neutralizing antibody (N=5). Data was analyzed using the paired T test; NS non significant, * p<0.05, ** p<0.01, *** p<0.001.

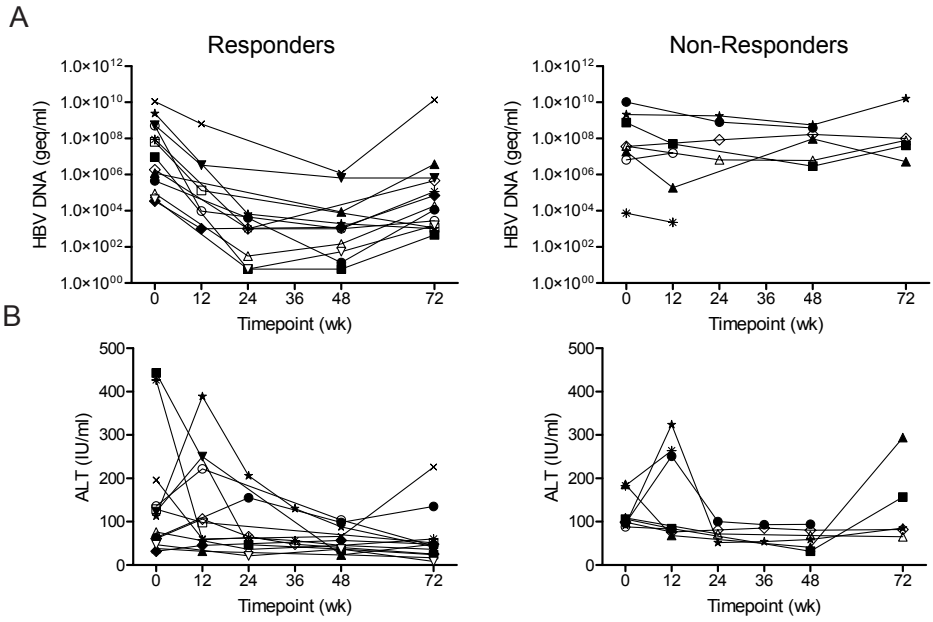

Fig. 3 Clinical outcome of PEG-IFN treatment of chronic HBV patients.
(A) Serum HBV-DNA levels and (B) serum ALT levels of responders (N=13) and non-responders (N=8) during 48 wk of PEG-IFN therapy. Indicated timepoints are baseline (wk 0), on therapy (wk 12, 24, 36), end of therapy (wk 48) and end of follow up (wk 72).

treatment (Fig. 4B: responders: 0 vs 12 weeks p=0.034). Although not significant, also pDC of non-responders expressed higher levels of CD86 (p=0.07). HLA-DR expression levels did not significantly differ during treatment compared to baseline in responders and non-responders. CD80 and CD40 could not be detected on circulating pDC before or during therapy (data not shown). Interestingly, PEG-IFN therapy had a drastic effect on pDC function. IFN-α production after TLR9 triggering diminished in both responder and non-responder groups, while response to TLR7 triggering highly increased during PEG-IFN therapy (Fig. 4C). These effects reversed to baseline levels 24 weeks post therapy and were not related to clinical outcome. Since all observed effects on pDC frequency, phenotype and function correlated only with PEG-IFN therapy and not with viral load or ALT levels, responders and non-responders were combined to study the effect of PEG-IFN therapy in more detail.

Differential response to TLR7 and TLR9 stimulation of pDC of chronic HBV patients on PEG-IFN therapy

Combining data from responders and non-responders, TLR7-induced IFN-α production by pDC increased 5-fold during PEG-IFN therapy as compared to baseline (Fig. 5A). In contrast to the stimulatory effect of IFN-α on TLR9-induced cytokine production by pDC exposed to IFN-α *in vitro*, exposure to IFN-α in vivo (during PEG-IFN therapy) resulted in a 3-fold reduction in TLR9-induced IFN-α production (Fig. 5B). In contrast to the additive effect of IFN-α in cultures prior to treatment, supplementary *in vitro* exposure to IFN-α during PEG-IFN therapy did not

enhance pDC function. Both effects on TLR7/9 triggering and on sensitivity to supplementary IFN-α reversed to baseline levels at 24 weeks post PEG-IFN treatment. PEG-IFN therapy had no significant effect on IFN-α production upon stimulation with HSV or influenza virus after four weeks (data not shown) or any other timepoint measured (Fig. 5C).

PEG-IFN therapy downregulates both TLR9-induced IFN-α and TNF-α production of pDC of chronic HBV patients

Response to TLR9 triggering was studied in more detail in a subset of patients during the first 12 weeks of PEG-IFN therapy. IFN-α production upon CpG-A, CpG-B and CpG-C stimulus diminished already after four weeks, as did the sensitivity to immune stimulation by IFN-α culture (Fig. 6A). PEG-IFN therapy also reduced the CpG-induced production of TNF-α, which was already visible after 4 weeks for CpG-A and after 12 weeks for CpG-B and CpG-C stimulation (Fig. 6B). Also in these experiments pDC lost sensitivity to the presence of IFN-α during culture already after 4 weeks of therapy. PEG-IFN therapy thus interferes with TLR9 response to all three classes of CpG, either directly or via an effect on other cell types within the unfractionated PBMC.

PEG-IFN therapy directly interferes with pDC function

To investigate whether PEG-IFN therapy directly affects pDC function, TLR7 and TLR9 triggering of pDC of chronic HBV patients prior to and during therapy was performed with purified pDC. Upon PEG-IFN therapy, purified pDC showed exactly similar responses to TLR7 and TLR9 stimuli as pDC present within PBMC. IFN-α and TNF-α production after TLR9 stimulation by CpG was lost during therapy, as was the sensitivity to the immune stimulating effects of supplementary IFN-α (Fig. 7A). TLR7 stimulation of pDC obtained from HBV patients on therapy led to three times more IFN-α producing pDC after 4 weeks and two times more after 12 weeks, compared to cells obtained at baseline. TNF-α was produced in unaffected high levels after TLR7 stimulation. All effects were reversed at 24 weeks post PEG-IFN therapy (data not shown).

PEG-IFN therapy causes differential regulation of TLR7 and TLR9 mRNA levels of pDC

To unravel the mechanism underlying the differential response to TLR7 and TLR9 triggering during therapy, the mRNA expression levels of TLR7 and TLR9 were investigated. In line with the functional data, RT-PCR analysis of purified pDC showed that PEG-IFN therapy significantly reduced TLR9 mRNA levels (Fig. 8). TLR7 mRNA levels in pDC were 1.3-fold augmented after 4 weeks of therapy, this increase normalized in half of the patients after 12 weeks, which was compatible with the Lox-triggered IFN-α production at 4 and 12 weeks of treatment. Although inter-individual changes exist regarding the regulation of TLR7 and TLR9 mRNA levels, no difference was found between responders and non-responders to PEG-IFN therapy. These results indicate a direct effect of PEG-IFN therapy on pDC function through differential regulation of TLR7 and TLR9 expression levels.

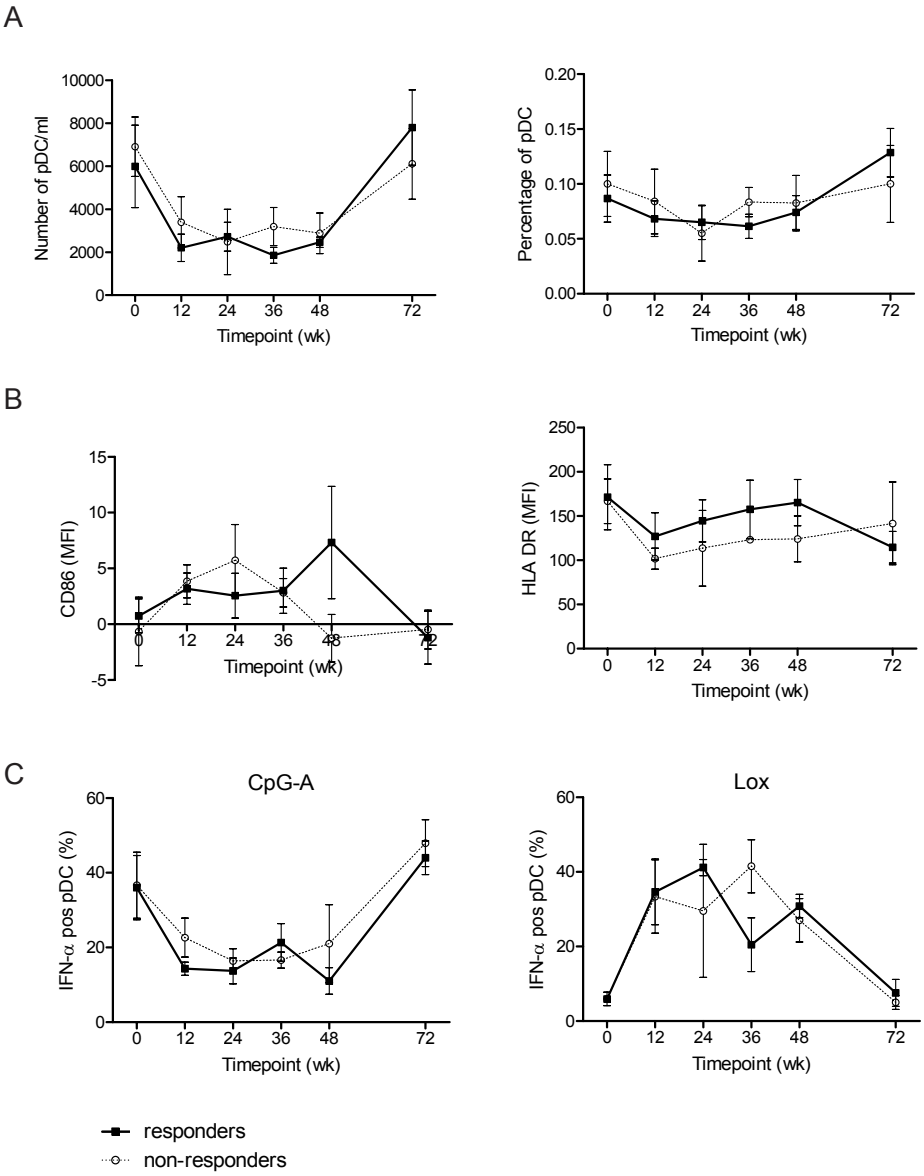

Fig. 4 PEG-IFN therapy affects numbers, phenotype and function of pDC in blood of chronic HBV patients. (A) Total and relative pDC numbers in whole blood samples of chronic HBV patients on PEG-IFN therapy determined by white blood cell count and flow cytometry. (B) Surface expression of HLA-DR and CD86 on CD123+BDCA4+ pDC of chronic HBV patients on PEG-IFN therapy. Values were normalized by subtraction of the MFI of the IgG control staining. (C) Number of IFN-α producing pDC after TLR9-triggering (CpG-A) and TLR7-triggering (Lox) obtained from blood of chronic HBV patients on PEG-IFN therapy. Data represent mean±SEM. Responders: N=7; non-responders: N=5 before therapy, N=4 at end of therapy (wk 48) and N=3 at end of follow up (wk 72).

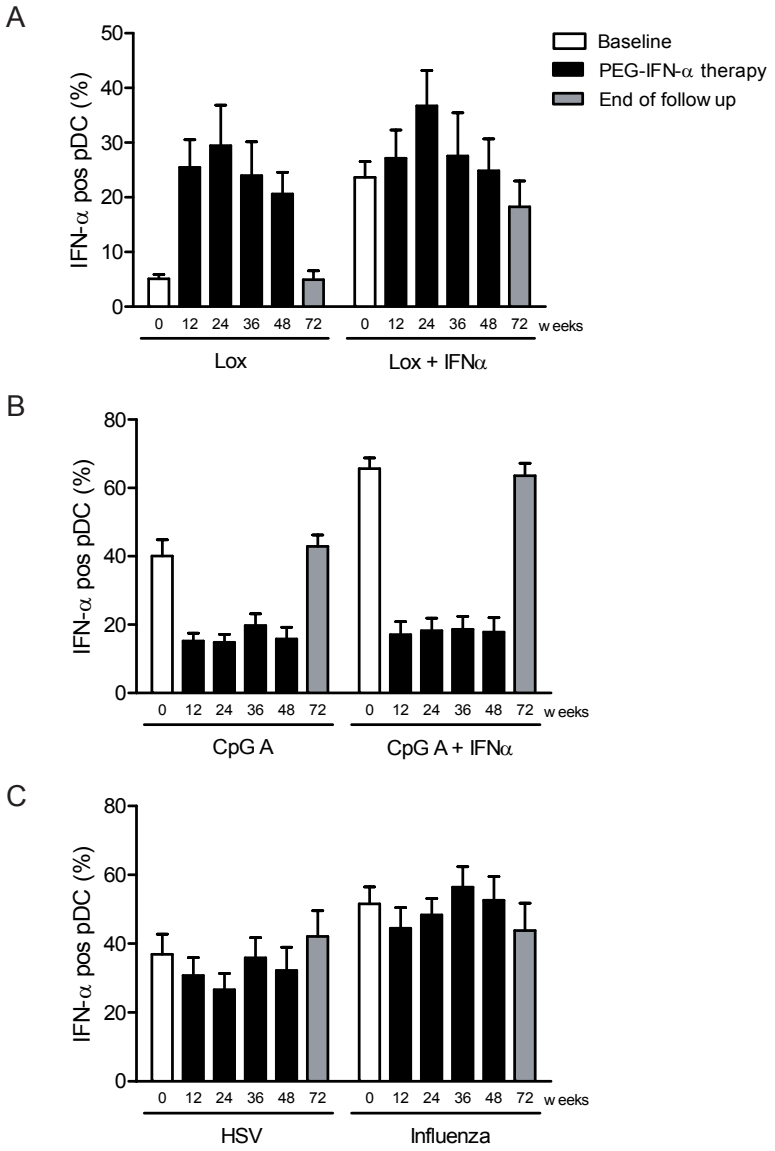

Fig. 5 IFN-α production after TLR7 and TLR9 stimulation of pDC of chronic HBV patients on PEG-IFN therapy. PBMC obtained from PEG-IFN treated chronic HBV patients were stimulated for 5 hr in the presence or absence of IFN-α and produced IFN-α was determined by flow cytometric analysis of CD123+BDCA4+ pDC. (A) Number of IFN-α producing pDC after Lox stimulation (N=12, wk 0 vs wk 12: p=0.0019). (B) Number of IFN-α producing pDC after CpG stimulation (N=17, wk 0 vs wk 12: p=0.0007). (C) Number of IFN-α producing pDC after stimulation with HSV-1 and influenza virus (N=12). Data represent mean±SEM and samples were analyzed using the paired T test.

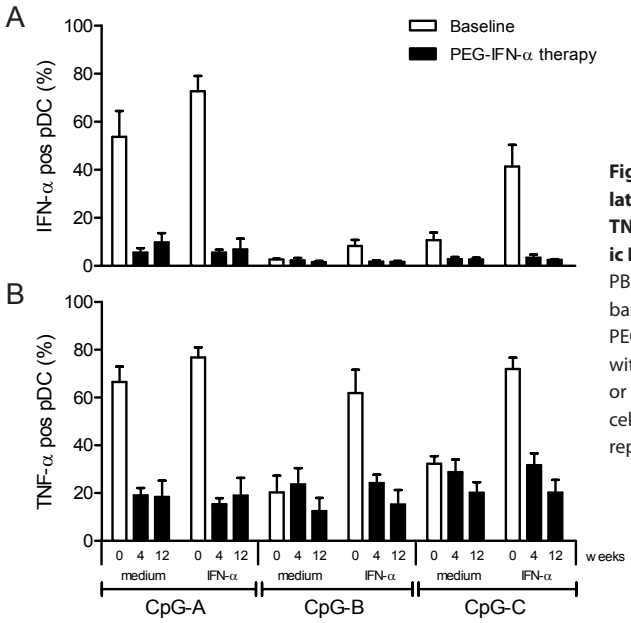

Fig. 6 PEG-IFN therapy downregulates both TLR9-induced IFN-α and TNF-α production of pDC of chronic HBV patients.
PBMC of chronic HBV patients at baseline and at 4 and 12 weeks of PEG-IFN therapy were stimulated with CpG-A, CpG-B and CpG-C with or without IFN-α to determine intracellular IFN-α (A) and TNF-α (B). Data represent mean±SEM; N=4.

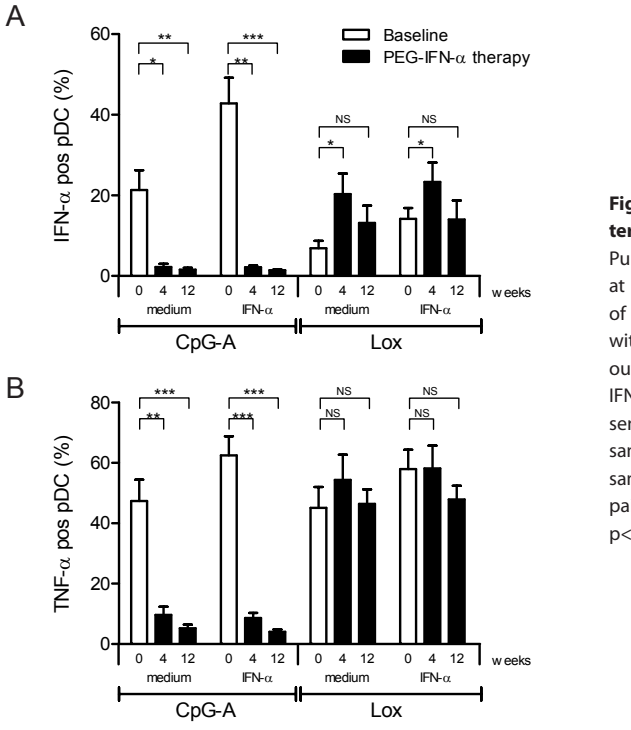

Fig. 7 PEG-IFN therapy directly interferes with pDC function.
Purified pDC of chronic HBV patients at baseline and at 4 and 12 weeks of PEG-IFN therapy were stimulated with CpG-A and Lox with or without IFN-α to determine intracellular IFN-α (A) and TNF-α (B). Data represent mean±SEM; N=7. On treatment samples were analyzed against the samples before treatment using the paired T test; NS non significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

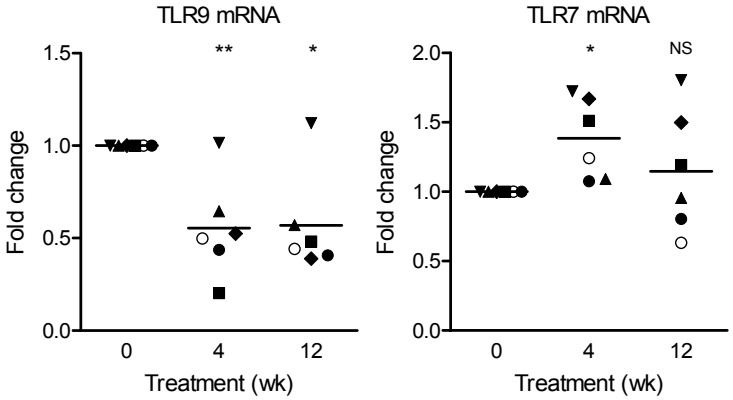

Fig. 8 PEG-IFN therapy directly interferes with pDC function by differential regulation of TLR7 and TLR9 mRNA levels.

RT-PCR analysis of purified pDC at baseline and at 4 and 12 weeks of PEG-IFN therapy. Data are normalized against expression of the reference gene Abl, the fold change compared to baseline of relative TLR9 and TLR7 mRNA levels are shown for 6 chronic HBV patients. On treatment samples were analyzed against the samples before treatment using the paired T test; NS non significant, * $p<0.05$, ** $p<0.01$.

DISCUSSION

PDC are key players in the anti-viral immune response. The strong initial type I IFN response upon viral infection, often considered as effective pDC activation, is lacking in acute HBV infection [11,12]. Moreover, patients chronically infected with HBV show functional deficits in pDC function [21]. Here we demonstrated that IFN-α increased pDC function with respect to the numbers of anti-viral cytokines-producing pDC upon TLR7 and TLR9 triggering *in vitro*, via the IFNAR-mediated positive feedback loop. In contrast, *in vivo* exposure to IFN-α enhanced TLR7-induced pDC function, but significantly decreased the response to TLR9 stimulation by upregulation of TLR7 and downregulation of TLR9 mRNA. IFN-α treatment *in vitro* had some positive effect on cell viability (not shown) and only showed a modest effect on pDC phenotype. Exposure to IFN-α *in vivo* led to a decrease in circulating pDC numbers which could be explained by either apoptosis or relocation of these cells. Apoptosis is not very likely since *in vitro* exposure to IFN-α slightly enhanced pDC survival; therefore migration seems the most plausible explanation. This would also explain the observed decrease in percentage of pDC within the CD45+ cell fraction. Only little PEG-IFN therapy-induced maturation was observed, which is likely underestimated in case of migration of activated pDC. Although IFN-α therapy provoked changes in pDC numbers, phenotype and function, none of these changes correlated with either viral load or serum ALT levels. We therefore conclude that the observed effects on pDC are directly caused by PEG-IFNα-2a therapy and are not predictive for treatment outcom. The rapid type I IFN response of pDC after detection of viral intrusion is supported by the IFNAR-mediated autocrine and paracrine positive feedback loop, leading to induction

of IFN-α and IFN-stimulated genes [22]. *In vitro* activation of the feedback loop by the presence of supplementary IFN-α enhanced the response to all three classes of CpG in this study. Independent of treatment outcome, we have shown that the IFN-α feedback loop is completely abolished by PEG-IFN therapy. This might be an important mechanism to prevent overzealous IFN-α production, which could lead to chronic inflammatory diseases. A simple explanation of this phenomenon would be the disappearance of IFNAR from the cell surface of pDC during of PEG-IFN therapy, but this was not observed (data not shown). Interference with IFN-α signaling might be responsible instead.

Using DNA microarrays, response to PEG-IFN-α and ribavirin combination therapy of chronic HCV patients has been studied. The number of genes, including known IFN-stimulated genes, that were up- or down-regulated by PEG-IFN and ribavirin combination therapy was fewer in patients with a poor response than in those with an intermediate or marked viral response, but no specific regulatory gene could be identified as responsible for this global difference. Interestingly, increased levels of TLR7 mRNA were observed in patients with intermediate or good response to therapy in this study [23]. In our small patient population, no such trend was observed. Recently, several reports have shown a correlation between PEG-IFN and ribavirin combination therapy outcome in HCV patients and activation of proteins of Jak/STAT signaling route: presence of STAT6 in pretreatment samples [24] and absence of suppressor of cytokine signaling 3 (SOCS3) in the liver prior to treatment [25] predict positive treatment outcome, while impaired phosphorylation of STAT1 is found in treatment non-responders [26].

Apart from the effect on IFN-sensitivity, this study shows that long term exposure to PEG-IFN interferes with CpG-induced pDC function, but does not significantly affect the response to viral stimuli. In contrast, Goutagny and colleagues showed a reduced capacity to release IFN-α after HSV-1 stimulation of pDC from HCV-infected patients on IFN-α and ribavirin combination therapy [27]. The additive effect of the immunomodulator ribavirin on pDC function might be one of the explanations for the observed difference compared to this study. Among the other differences are the stimulation time, the detection method of IFN-α production and the type of chronic viral infection. The reduced HSV-induced IFN-α production might also simply be the result of reduced numbers of pDC within the PBMC population, a phenomenon we circumvented by using intracellular IFN-α detection of stimulated pDC.

The fact that PEG-IFN-induced upregulation of TLR7 mRNA does not lead to enhanced response after influenza virus stimulus, might be explained by the higher initial response to influenza virus compared to the synthetic ligand Lox. This high level response indicates the involvement of other cellular receptors triggered by the virus. Similarly, the presence of other viral cellular receptors might explain why the decreased TLR9 mRNA levels coincide with diminished IFN-α response to CpG- but not HSV-triggering.

Next to the here reported differences in mRNA levels of TLR7 and TLR9 inflicted by PEG-IFN therapy, it would be informative to study the actual protein levels of these receptors in pDC. Currently, this cannot be achieved due to technical limitations. TLR7 and TLR9 reside in

endosomal compartments of pDC and so far no reliable flow cytometry staining can be performed. Alternatively, cell pellets of pDC could be analysed by Western blot, but this requires an impractical amount of cells that can not be obtained from patients on therapy.

In line with previous studies, we observed that the three main classes of CpG oligonucleotides, CpG class A, B and C, have different patterns of immune activation. CpG-A induces more IFN-α, partially regulated via activation of the IFNAR-mediated autocrine feedback loop [28], which is not activated by CpG-B [29]. Here, we observed that total cytokine secretion after CpG-A stimulus is abrogated during PEG-IFN therapy since TLR9 levels are downregulated *and* functionality of the IFNAR-mediated feedback loop is lost. Cytokine secretion after CpG-B is independent of the IFNAR-mediated feedback loop and reduction is solely the result of reduced TLR9 levels. Indeed, CpG-B-mediated cytokine secretion is reduced to a lesser extent than CpG-A-mediated secretion. As expected, results of CpG-C stimulus are an intermediate of the effect of CpG-A and CpG-B stimuli.

In conclusion, PEG-IFN therapy influenced total number and function of pDC of chronic HBV patients. In contrast to *in vitro* exposure to IFN-α which upregulates response to both TLR7 and TLR9 triggering, continuous exposure to IFN-α *in vivo* downregulates TLR9-and upregulates TLR7-induced pDC function. Although it might not seem particular relevant to investigate IFN-α production by pDC in patients who are receiving high amounts of PEG-IFN, analysis of IFN-α and TNF-α production is informative for pDC function in general, especially since NK or T cell crosstalk are difficult to study with such limited cell numbers. It is tempting to speculate that a diminished TLR9-induced pDC function in patients chronically infected with a DNA-virus negatively contributes to response to treatment, which would explain the relative poor response to PEG-IFN therapy of chronic HBV patients in comparison to HCV patients. Although no relation with clinical outcome, these data will aid in a better understanding of the anti-viral immune response induced with PEG-IFN therapy and thereby help in design novel treatment strategies for chronic HBV infections.

ACKNOWLEDGEMENT

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CHAPTER 8

Discussion

Myeloid dendritic cells in Hepatitis B virus infection

This thesis focused on the role of dendritic cells (DC) in chronic HBV infection. Due to their critical role in anti-viral immunity, viruses, such as HBV, might have evolved to interfere with the function of DC to escape the host immune response. It has been demonstrated that DC of chronic HBV patients display a less immunogenic function compared to DC of healthy controls [1-4]. The functional experiments in these studies focussed on the capacity of DC to stimulate allogeneic T cells, measuring primarily CD4+ T cell proliferation. Next to this general DC deficit, in chapter 2 it is demonstrated that myeloid DC (mDC) are also impaired in HBV-specific CD8+ T cell proliferation. In chapter 3 a possible explanation for this phenomenon is given by showing that mDC actively internalize HBsAg and that the presence of either purified HBsAg or intact viral particles (in concentrations comparable to measured amounts in patient sera [5]) during mDC maturation gives rise to mDC with a significantly less immunogenic phenotype and function as demonstrated by the reduced expression of co-stimulatory molecules and decreased T cell stimulatory capacity [6]. HBV, but not HBsAg, also significantly decreased their ability to produce IL-12. This is in accordance with data from HBV transgenic mice, used as a model for chronic HBV carriers, which produce HBsAg, HBeAg and the viral genome. These mice show low immune efficiency, as defined by decreased overall specific antibody responses and reduced T cell stimulatory capacity of DC [7]. Also in an HBsAg/HLA-A2 humanized transgenic mouse model, high serum levels of HBsAg were able to induce T cell tolerance to HBsAg [8]. These data suggest that HBsAg, either as subviral particles or as part of the viral envelop, is at least partially responsible for the impaired mDC function observed in chronic HBV patients. This is supported by the fact that viral load reduction by the nucleotide analogue adefovir strongly increased the capacity of mDC of chronic HBV patients to produce IL-12 and to activate allogeneic T cells *ex vivo* [4]. This observation might be a direct effect of the decreased viral load and/or circulating HBsAg levels in these nucleotide analogue-treated patients. However, it cannot be excluded that other immune parameters are modulated by the lower viral levels, such as reduced numbers of circulating regulatory T cells [9], which could be the *result,* as well as the *cause* for improved mDC function [10,11].

Although the envelope proteins of HBV are glycosylated, the active time-dependent uptake of HBsAg was not mediated through DC-SIGN (chapter 4, [12]). In chapter 5 the role of the mannose receptor in the binding and uptake of HBsAg is discussed. In circulating blood mDC the cell surface expression of the mannose receptor was generally very low, this was reflected by the lack of HBsAg uptake and the relative low capacity to take up dextran-FITC as a model substrate for lectin-mediated endocytosis. The presence of HBsAg-positive mDC was indeed demonstrated only sporadically in blood of chronic HBV patients, which positively correlated to expression of the mannose receptor. Hepatic mDC of chronic HBV patients were more frequently involved in the recognition and uptake of HBV subviral particles, which is likely mediated through the mannose receptor [13]. In contrast to pathogen recognition by e.g. Toll like receptors (TLR), interaction with the mannose receptor does not induce DC maturation [14].

Thus the mannose receptor-mediated HBsAg uptake in the absence of a TLR signal may lead to HBV/HBsAg-specific tolerance. Indeed, in HBV transgenic mice, liver-derived DC were found to be impaired in the capability to stimulate naive T cells and produce cytokines, including IL-12 and IFN-γ [15].

Since the mannose receptor is known to be expressed on macrophages, Kupffer cells in the liver biopsies of the same chronic HBV and control patients were also analysed by flow cytometry (Fig. 1A). The mannose receptor was present on approximately 7.5% of all CD14+ Kupffer cells, ranging from 0.7 to 15.7%. HBsAg could be detected on or within 3.6% of all Kupffer cells, ranging from 0.3 to 11.8%, as observed for mDC. Presence of HBsAg and MR expression of Kupffer cells showed a trend towards positive correlation (p=0.09, Fig. 1B). Kupffer cells can induce tolerance towards phagocytosed antigens by deletion of T cells through induction of apoptosis [16]. These results further imply that antigen presenting cells in the immunotolerant environment of the liver play a role in tolerance towards HBV.

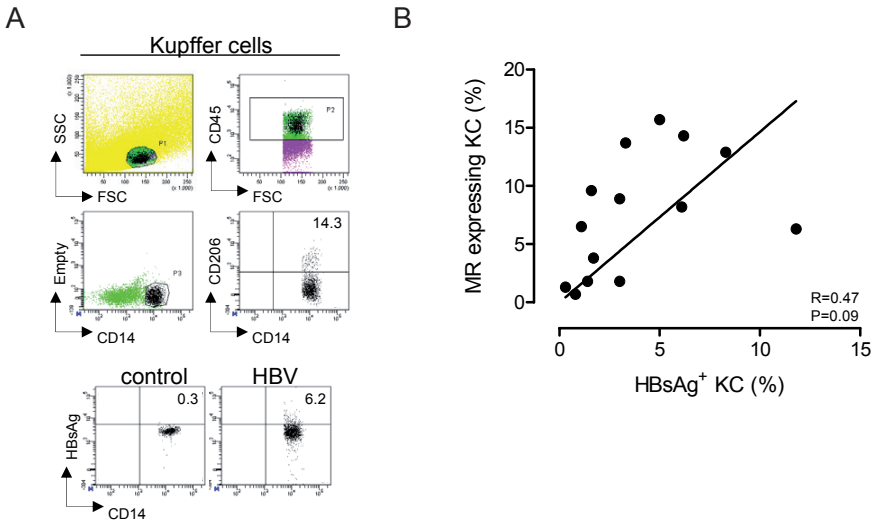

Fig. 1 Presence of HBsAg and MR expression level in Kupffer cells of chronic HBV patients.
Liver biopsy samples of patients with chronic HBV and control patients with non-HBV related liver diseases were measured by flow cytometry after staining for anti-CD45, anti-CD14, anti-CD206 (MR) and anti-HBsAg. (A) Kupffer cells are identified as CD45+CD14+, within this population the percentage HBsAg positive cells was determined as shown for a representative control and HBV patient. (B) The correlation between HBsAg positivity and presence of MR on Kupffer cells of 14 chronic HBV patients was determined with the Spearman test (r=0.47, p=0.09). MR/CD206=mannose receptor; KC=Kupffer cell.

In healthy individuals, the immune response after pathogen invasion combines optimal pathogen clearance with minimal damage to the host. The immunosuppressive cytokine IL-10 is important in the maintenance of this balance through inhibition of cellular immune responses: IL-10 inhibits DC function (prevention of DC maturation, blocking of pro-inflammatory cytokine production and co-stimulatory molecules expression) leading to effector T cell unresponsiveness

and differentiation of regulatory T cells [17]. In case the balance is lost, underproduction of IL-10 may contribute to inflammatory diseases such as asthma [18] and Crohn's disease [19]. Overproduction of IL-10 is thought to be an important factor in persistence of viral infections [20] and elevated serum IL-10 levels have been found in HIV [21], HCV [22] and HBV patients [23]. In a mouse model for chronic viral infections, significant upregulation of IL-10 by antigen-presenting cells was reported, leading to impaired T cell responses. Therapeutic administration of an antibody that blocks the IL-10 receptor restored T cell function and eliminated viral infection in the chronically infected wild type mice. Viral infection of IL-10-deficient mice resulted in the maintenance of effector T cell responses, elimination of virus and development of antiviral memory T cell responses [24]. The role of IL-10 in the chronicity of HBV infections is getting increasingly more attention lately. A recent study showed an induction of IL-10 during the acute phase of HBV infections, which was accompanied by increased levels of HBV vireamia and attenuation of NK and T cell responses [25]. HBcore triggered IL-10 production by monocytes and T cells after stimulation of chronic HBV patient-derived PBMC and frequencies of IL-10 producing monocytes and T cells were significantly higher in patients with high HBV viral load and ALT levels [26,27]. Neither HBV particles nor HBsAg directly induced IL-10 production by isolated mDC *in vitro* (chapter 3 [6]). Nevertheless, using TNF-α and IL-1β as stimulation we demonstrated the presence of more IL-10-producing mDC in chronic HBV patients compared to healthy controls; IL-10 neutralization resulted in restoration of HBV-specific CD8 T cell expansion (chapter 2). Earlier studies of our group showed that adefovir-induced viral load reduction reduced the number of regulatory T cells in the circulation of chronic HBV patients. However, this increased response was not related to decreased numbers of IL-10 producing cells [9]. The lack of decrease in IL-10 producing cells might be one of the reasons why viral load reduction by nucleos(t)ide analogues does not often lead to sustained anti-viral response.

Plasmacytoid dendritic cells in Hepatitis B virus infection

Upon detection of signs of viral invasion, plasmacytoid dendritic cells (pDC) produce large amounts of the anti-viral cytokine IFN-α. Detection of viral genetic material and/or viral replication intermediates occurs in the endosomal compartments through TLR; single-stranded RNA triggers TLR7 and double-stranded DNA containing CpG motifs triggers TLR9 [28]. The rapid type I IFN response of pDC after detection of viral intrusion is guaranteed by the IFN-α autocrine and paracrine positive feedback loop. Interaction between IFN-α and IFN-α/β receptor (IFNAR) leads to activation of Jak/STAT signalling, phosphorylated STAT proteins then translocate to the nucleus where they assemble with the DNA-binding protein IFN-stimulated gene factor (ISGF)3γ to form the ISGF3 complex. ISGF3 functions as a transcription factor for genes containing an IFN-stimulated response element motif in their promoter region. Among the genes induced by the IFN-α feedback loop is IRF7. Translation and activation of IRF7 proteins in response to IFN are thought to be required for the induction of IFN-α gene transcription [29-31].

In this virus-host interaction, it is in the interest of the virus to secure a niche for its existence and escape the host anti-viral response [32]. HBV seems to have indeed found a way to escape the primary type I IFN response of the host. Although HBV is a DNA virus which replicates through an RNA intermediate, no IFN-α production is triggered after incubation of HBV with pDC *in vitro* (chapter 6). Moreover, HBV infected chimpanzees do not demonstrate induction of IFN-α or IFN-β during the acute phase of infection [33]. It is difficult to study the early events of acute HBV infection in humans, since patients mostly present after onset of clinical symptoms (nausea and jaundice) observed 10-12 weeks after infection [34]. Bertoletti and Gehring made the interesting observation that the lack of early symptoms such as fever and malaise in HBV-infected patients is indirect evidence of the defective type I IFN response during the acute phase of HBV infection [35]. One of the proposed mechanisms by which HBV escapes the first line defence of the host, is the localization of HBV replication within viral nucleocapsid particles, which renders viral RNA and DNA replication intermediates protected from recognition [33,35]. A simple explanation for the lack of IFN-α production after HBV exposure could also be that pDC are just not capable of binding and internalizing HBV. No signs of HBV replication within pDC have been reported, but since HBV DNA has been found on or within circulating pDC they seem at least capable of HBV binding [1,36]. A recent study proposed that HBsAg interacts with pDC through binding of the pDC specific C-type lectin BDCA2 [37]. In chapter 6 we showed that, although limited, HBsAg is indeed able to bind to non-stimulated pDC. However, the role of BDCA2 in HBsAg binding remains controversial. In our hands, BDCA2 is highly expressed on non-stimulated pDC and is downregulated upon stimulation with CpG, whereas the binding of HBsAg was strongly increased. In addition, TLR7-stimulated pDC showed a similar expression of BDCA2, but did hardly bind HBsAg, thereby indicating that BDCA2 cannot be the exclusive cellular HBsAg receptor on pDC.

Another mechanism of viral escape is the proposed direct interference of HBV with the IFN-α production or signalling pathway. TLR9-induced IFN-α production by pDC requires mTOR activation for interaction between TLR9 and MyD88 and subsequent IRF-7 phosphorylation and nuclear translocation in order to activate IFN-α gene transcription [38]. Chapter 6 described that the direct inhibitory effect of HBV on TLR9 triggering, leading to impaired pDC function and activation, is mediated through reduction of IRF-7 phosphorylation. In concordance it was recently shown that HBsAg directly reduced TLR9-mediated IFN-α production, by inhibition of IRF7 expression and nuclear translocation [37]. Several other reports describe the inhibition of IFN-α signalling by HBV at an upstream signal level using different cell lines. The polymerase protein of HBV has been shown to inhibit IFN-α induced activity of the MyD88 promoter at the STAT binding site, through inhibition of STAT-1 nuclear translocation [39]. Activation of STAT-1 was also found to be inhibited by HBV both *in vitro* and in liver biopsies of chronic HBV patients [40]. All together these data suggest that HBV may escape immunity via ineffective pDC activation and/or active inhibition of pDC function. Disruption of the IFN-α signaling pathway is not unique to HBV: many other viruses exploit it to reduce antiviral IFN responses [41]. HCV proteins

are capable of selectively degrading STAT-1, to reduce accumulation of phosphorylated STAT-1 in the nucleus, and to interfere with specific molecules of IFN-α signaling pathways activated upon recognition of viral RNA [11,42]. The filoviruses Ebola and Marburg encode proteins to interfere with IRF3 and IRF7 activation. In addition, Ebola virus prevents nuclear import of activated STAT-1, while Marburg virus acts more upstream by preventing STAT-1 phosphorylation [43]. Paramyxoviruses directly interact with STAT-1; this sequestration prevents STAT-1 activation and thereby blocks IFN-α signalling [44]. More knowledge on these evasive viral strategies which potently disrupt the antiviral response of the host will lead to better strategies (e.g. therapeutic drugs or targeted vaccines) to control and prevent viral infection.

In chronic HBV patients, the impaired IFN-α signaling pathway leads to impaired IFN-α secretion as shown in several studies comparing pDC function in chronic HBV patients and healthy controls [1,45-48]. It has been suggested that downregulation of TLR9 levels in chronic HBV patients contributes to the impaired IFN-α production [48]. The proposed functional pDC impairment has been questioned by Tavakoli *et al*. In the comparison of CpG-induced IFN-α production of pDC of chronic HBV patients versus healthy individuals, they observed such considerable variation between donors that no significant difference between the two groups was found [36]. Indeed we feel that some caution in the interpretation of the studies on impaired IFN-α production by pDC of chronic HBV patients is at place. We have observed not only large inter-person variability, but also an evident effect of age on CpG-induced IFN-α production (Fig. 2).

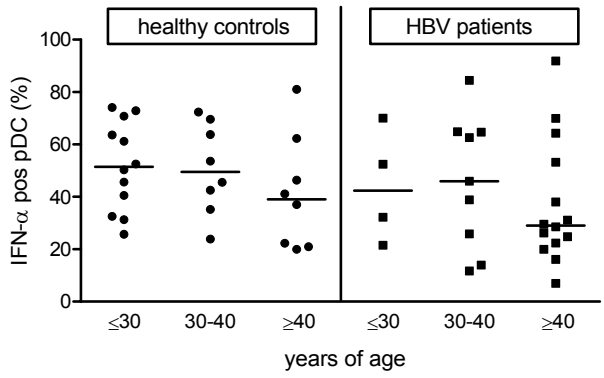

Fig. 2 Inter-person variability and the effect of age on CpG-induced IFN-α production of pDC of healthy individuals versus chronic HBV patients.
Total peripheral blood mononuclear cells were isolated from healthy controls or chronic HBV patients. Cells were stimulated with CpG for 5 hrs and intracellular IFN-α was measured by flow cytometry.

In detailed observation of patient and control characteristics, we found that the median age is higher and/or the range in age is broader of all patient populations reported [1,10,45,47,48], excluding the study with pediatric patients [46]. Age-related changes in human blood dendritic cell populations are getting more and more attention and we strongly recommend to carefully consider these effects in future study design [49-53].

The absence of IFN-α induction early in HBV infection, combined with the impaired IFN-α production during the adaptive immune response later in the infection, might contribute to the susceptibility of chronic HBV patients to PEG-IFN-α therapy. It is known that IFN-α has a direct inhibitory effect on HBV replication. In HBV transgenic mouse models, both IFN-α/β inhibit HBV replication through interference with the assembly of viral nucleocapsids [54]. Gene expression analysis of IFN-induced genes in HBV transgenic mouse livers revealed several cellular factors that are associated with IFN-α induced suppression of HBV replication, including components of the immunoproteasome, ubiquitin-like proteins and several GTP-binding proteins [55]. Next to this direct anti-viral effect, PEG-IFN-α therapy also has an immunomodulatory effect: IFN-α activates macrophages, natural killer cells and T lymphocytes allowing further priming of anti-viral immunity [56]. Nevertheless, only approximately 30% of patients remain in the immune control phase after discontinuation of therapy [57]. The vast majority of patients might suffer from IFN-α-induced side effects, such as flu-like symptoms, cytopenia and psychiatric adverse events, without clinical benefit [58]. In chapter 7 we showed that PEG-IFN-α-2a therapy provoked changes in pDC number, phenotype and function but could not overcome the initially observed impaired IFN-α production. Instead, PEG-IFN-α-2a interfered with the IFNAR feedback loop diminishing the sensitivity of pDC to IFN-α. Preliminary results on flow cytometric analysis of pDC samples before and during PEG-IFN-α-2a therapy ruled out that down regulation of IFNAR on the cell surface is responsible for this effect. Possible interference at other levels of the IFN-α signalling is currently under investigation. In contrast to *in vitro* exposure to IFN-α which upregulates response to both TLR7 and TLR9 triggering, continuous exposure to IFN-α *in vivo* downregulates TLR9-and upregulates TLR7-induced pDC function. Unfortunately, none of these changes correlated with viral load or ALT levels and are thus not predictive for treatment outcome.

Since sustained viral response to PEG-IFN therapy is obtained only in a minority of chronic HBV patients, more effective immune modulation might enhance the sustained response rate. Therefore we compared DC function of patients on PEG-IFN monotherapy, with patients on PEG-IFN and ribavirin combination therapy. Ribavirin is a nucleoside analogue with putative immunomodulatory capacities and addition to PEG-IFN doubled the sustained response rate in chronic HCV patients [59]. This effect of ribavirin is attributed to a better restoration of the immune response, but the underlying mechanism is not known. Compared to PEG-IFN alone, which only induced a transient activation of pDC as demonstrated by increased expression of CD86 (chapter 7), combination therapy induced a much higher upregulation of CD80, CD86 as well as CD40 that sustained the whole treatment period (Fig. 3). At week 72, these parameters reversed or were slightly decreased compared to baseline. pDC function, as examined by CpG-induced IFN-α production, was dramatically reduced during treatment, but not different between the groups. The immunological differences between the treatment groups did not translate into a clinical effect since viral load decline and virological (HBV DNA $<10^4$ at week 72), and biochemical responses (normalized ALT at week 72) were comparable in the two arms.

In conclusion, compared to PEG-IFN monotherapy, addition of ribavirin significantly enhances and prolongs DC activation, which may favour anti-HBV specific immunity but is not sufficient to improve virological and biochemical response rates in chronic HBV patients. Currently, the effects on other cell subsets, mDC, T cells and natural killer cells, are under investigation.

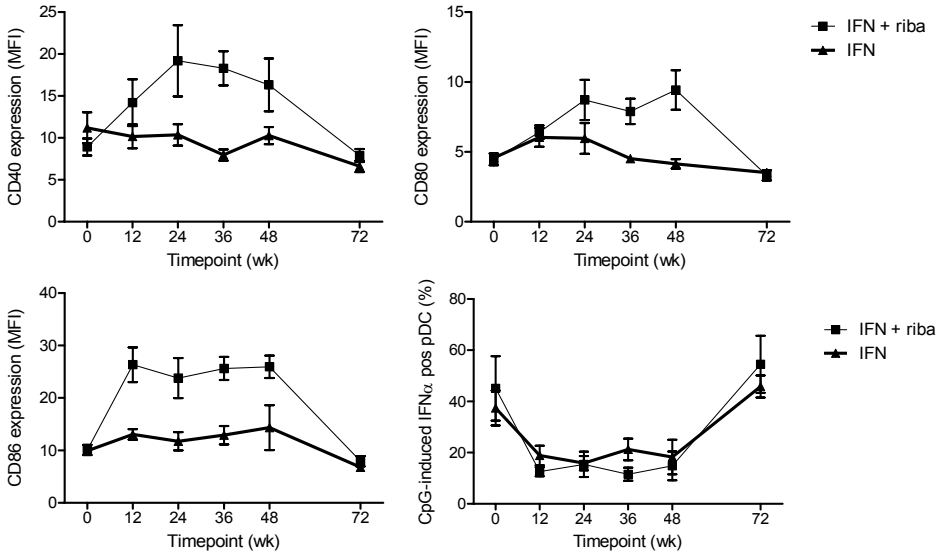

Fig. 3 Effect on pDC phenotype and function of PEG-IFN-α-2a monotherapy versus PEG-IFN-α-2a and ribavirin combination therapy of chronic HBV patients.

14 HBeAg-negative CHB patients who were randomized to receive PEG-IFNα-2a 180 mg weekly plus placebo (n=8) or ribavirin 1000-1200 mg daily (n=6) for 48 weeks, donated peripheral blood at baseline, at 12, 24, 36 and 48 weeks of treatment and at 24 weeks after treatment. Plasmacytoid DC (BDCA4+) were analyzed for expression of CD40, CD80 and CD86 by flow cytometry. To determine pDC function, cells were stimulated with CpG for 5 hrs and intracellular IFN-α was measured by flow cytometry. Baseline characteristics were comparable between the two arms, 11/14 male, age was 41.8±3.3 yrs (mean±SEM), HBV DNA was 9.6±3.9x106 IU/ml, ALT level was 145±37 IU/L and also DC parameters did not differ.

CONCLUDING REMARKS

This thesis focussed on the role of dendritic cells in chronic HBV. HBV surface antigens can directly interfere with both mDC and pDC function, although the effects are not of such a kind that they can solely account for the chronicity of HBV infections. The fact that chronic HBV patients do not frequently suffer from other opportunistic infections underlines this observation. Immunology is much like life itself: it is all about balance. Instead of one cell type being responsible, slight disturbances in functionality of all immune cell subsets, DC, natural killer cells and T cells, collectively result in tipping over the balance towards a chronic infection. Ongoing technical advantages make it possible to study the immune response at the site of infection: the liver. A start is made with this thesis to locally observe the phenotype of liver dendritic cells

that are contributing to the chronic infection and already important differences with circulating dendritic cells were found. It is my prediction that future studies on the functionality of liver immune cells will reveal even more drastic changes, so the key to resolving chronic HBV infections lies in its origin: the liver.

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Nederlandse samenvatting

Wereldwijd zijn er meer dan 400 miljoen mensen met een chronische Hepatitis B virus (HBV) infectie. Het gevaar van deze chronische besmetting is het optreden van leverschade met een verhoogd risico op levercirrose, leverfalen en leverkanker. Een goed functionerend immuunsysteem is in staat om een HBV infectie op te ruimen, maar in ongeveer 10 procent van de geïnfecteerde mensen gebeurt dit niet of niet voldoende, waardoor de acute infectie overgaat in een chronische infectie. De precieze oorzaak hiervan is onbekend. Uit voorafgaand onderzoek van het MDL-laboratorium naar dendritische cellen (DC), het celtype dat de anti-virale immuunrespons opstart en reguleert, is gebleken dat de functie van verschillende DC subtypes van chronische HBV patiënten afwijkt ten opzichte van gezonde controles. Naast een beschreven verminderde capaciteit van myeloïde DC (mDC) om CD4 T cellen te stimuleren, wordt in hoofdstuk 2 beschreven dat mDC van HBV patiënten minder goed in staat zijn om HBV-specifieke CD8 T cellen te activeren, wat een effectieve anti-virale immuunrespons belemmert. Neutralisatie van het anti-inflammatoire cytokine IL-10 herstelt het vermogen van DC om CD8 T cellen te activeren. Hieruit blijkt dat IL-10 mogelijk een belangrijke rol speelt in de ontwikkeling van een chronische HBV infectie. Tot dusver is niet bekend of de verminderde DC functie veroorzaakt wordt door een directe interactie tussen de DC en het virus, of door de achterliggende chronische leverontsteking. In hoofdstuk 3 is de interactie tussen mDC en HBV onderzocht en is vervolgens gekeken naar de gevolgen van deze interactie voor de anti-virale functie van mDC. Opname van HBV of HBV oppervlakte eiwitten leidt direct tot een functie afname van mDC. De interactie tussen HBV en de DC loopt echter niet via de welbekende pathogeen receptor DC-SIGN (hoofdstuk 4). De mannose receptor speelt wel een rol in de opname van HBV oppervlakte eiwitten door mDC. Uit onderzoek van bloed en leverbiopten van chronische HBV patiënten blijkt dat deze interactie voornamelijk in de lever plaatsvindt (hoofdstuk 5). Een ander subtype DC is de plasmacytoïde DC (pDC), die een belangrijke rol spelen bij de anti-virale afweer door de productie van interferon-α (IFN-α). In hoofdstuk 6 wordt beschreven dat HBV een directe negatieve invloed heeft op de IFN-α productie. Met name de IFN-α productie na triggering van de pathogeen receptor TLR9 wordt geremd in aanwezigheid van HBV, door een direct negatief effect op de IFN-α signaleringsroute. In vitro leidt IFN-α behandeling tot een verbetering van pDC functie (hoofdstuk 7). IFN-α therapie van chronische HBV patiënten leidt tot veranderingen in het aantal pDC, het fenotype en de functie. De IFN-α respons na stimulatie van TLR7 en TLR9 neemt respectievelijk toe en af, door regulatie van TLR7 en TLR9 mRNA levels. Bovendien verdwijnt de gevoeligheid van pDC voor de aanwezigheid van IFN-α, mogelijk door interferentie met de IFN-α signaleringsroute.

Samengevat tonen we met dit proefschrift aan dat HBV een negatief effect heeft op de functie van de twee belangrijke DC subtypes, mDC en pDC. Gecombineerd met specifieke individuele eigenschappen van de gastheer en de negatieve effecten van HBV op andere cellen van het immuun systeem, kan dit de balans laten doorslaan van een effectieve opruiming van HBV naar een chronische HBV infectie.

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Curriculum vitae

Marjoleine Louise Op den Brouw werd op 24 augustus 1978 geboren in Dordrecht. Zij behaalde in 1996 haar VWO diploma aan het Develsteincollege in Zwijndrecht. In datzelfde jaar startte zij met haar studie Moleculaire Wetenschappen aan de Wageningen Universiteit. In de doctoraalfase is haar wetenschappelijke interesse gewekt tijdens een afstudeeronderzoek aan het Laboratorium voor Virologie, waar ze onder begeleiding van dr. Wilfred IJkel en prof.dr. Rob Goldbach vier ORFs van het genoom van het Spodoptera exigua multicapsid nucleopolyhedrovirus gekarakteriseerd heeft. Hierna heeft ze onder begeleiding van dr. Joop Jansen onderzoek gedaan naar de pathogenese van acute promyelocytaire leukemie, aan het Hematologisch Laboratorium van het UMC St Radboud te Nijmegen. Om haar ervaring in het wetenschappelijk onderzoek verder uit te breiden heeft ze een extra stage van een jaar verricht aan de Harvard Medical School in Boston. Onder begeleiding van dr. Marianne Boes en prof.dr. Hidde Ploegh heeft ze onderzoek verricht naar antigeen presentatie door MHC class II moleculen met behulp van een GFP-MHC class II muizenmodel. Na het behalen van haar doctoraalexamen in 2002, heeft zij anderhalf jaar onderzoek gedaan aan genregulatie in Drosophila melanogaster op de afdeling Moleculaire Celbiologie van het LUMC in Leiden, onder begeleiding van dr. David Baker. In 2004 is zij gestart met haar promotieonderzoek op de afdeling Maag-, Darm- en Leverziekten van het Erasmus MC. Onder leiding van promotor prof.dr. Harry Janssen en co-promotor dr. Renate van der Molen, later opgevolgd door dr. Andrea Woltman, heeft zij onderzoek gedaan naar het Hepatitis B virus waarvan de resultaten in dit proefschrift beschreven staan. Een gedeelte van dit onderzoek heeft zij uitgevoerd op de laboratoria van dr. Theo Geijtenbeek (Moleculaire Celbiologie en Immunologie, VUMC, Amsterdam), Mark van Roosmalen (Biomérieux, Boxtel) en prof.dr. Ulrike Protzer (Instituut Medische Microbiologie, Keulen, Duitsland). Sinds juni 2009 is zij werkzaam bij Gilead Sciences als medical science liaison op het gebied van virale infectieziekten.

Publications

Op den Brouw ML, Binda RS, Geijtenbeek TBH, Janssen HLA and Woltman AM (2009) The mannose receptor acts as hepatitis B virus surface antigen receptor mediating interaction with intrahepatic dendritic cells. Virology 393(1): 84-90.

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Portfolio

Conferences

2009 Enhanced and prolonged activation of dendritic cells in chronic hepatitis B patients treated with a combination of PEG-IFN and ribavirin does not improve response to therapy compared to standard PEG-IFN therapy.
Annual meeting of the American Association for the study of Liver Diseases, Boston, USA (poster)

2008 The mannose receptor as a putative hepatitis B virus receptor regulating intrahepatic dendritic cell function.
Annual meeting of the American Association for the study of Liver Diseases, San Francisco, USA (Presidential Poster of Distinction)

PEG-IFNα2a therapy of chronic HBV patients alters plasmacytoid dendritic cell function by affecting TLR7 and TLR9 levels.
Annual meeting of the American Association for the study of Liver Diseases, San Francisco, USA (poster)

PEG-IFNα-2a therapy of chronic HBV patients alters the endogenous TLR-induced IFNα production by plasmacytoid dendritic cells.
Annual meeting of the Dutch Society of Gastroenterology and Hepatology, Veldhoven, the Netherlands (poster)

Internalization route of hepatitis B surface antigen by myeloid dendritic cells is dependent on HBV vaccination status.
Annual meeting of the Dutch Society of Gastroenterology and Hepatology, Veldhoven, the Netherlands (poster)

2007 Trimming of branched oligosaccharide structures on HBV inhibits DC-SIGN binding.
Annual meeting of the American Association for the study of Liver Diseases, Boston, USA (poster)

Trimming of branched oligosaccharide structures on HBV inhibits DC-SIGN binding.
International Meeting of the Molecular Biology of Hepatitis B viruses, Rome, Italy (oral)

PEG-IFNα-2a therapy of chronic HBV patients alters the endogenous TLR-induced IFNα production by plasmacytoid dendritic cells.
International Meeting of the Molecular Biology of Hepatitis B viruses, Rome, Italy (poster)

2006 Impaired IFNα production in patients with chronic HBV: Effect of IFNα treatment *in vitro* and *in vivo*.
Annual meeting of the Dutch Society of Immunology, Noordwijkerhout, the Netherlands (poster)

Impaired IFNα production of chronic HBV patients: exogenous IFNα restores endogenous IFNα levels.
Annual meeting of the American Association for the study of Liver Diseases, Boston, USA (poster)

Impaired IFNα production of chronic HBV patients: exogenous IFNα restores endogenous IFNα levels.
International Meeting of the Molecular Biology of Hepatitis B viruses, Vancouver, Canada (poster)

2005 Hepatitis B surface protein has an immunomodulatory effect on mDC.
Annual meeting of the Dutch Society of Immunology, Noordwijkerhout, the Netherlands (poster)

Functional impairment of mDC in chronic HBV patients: the role of HBV proteins.
Annual meeting of the American Association for the study of Liver Diseases, San Francisco, USA (poster)

Functional impairment of mDC in chronic HBV patients: the role of HBV proteins.
International Meeting of the Molecular Biology of Hepatitis B viruses, Heidelberg, Germany (poster)

Courses

2006 Medical statistics and SPSS

Memberships

2006 Member of the Dutch Society of Hepatology
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