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NATURAL KILLER CELLS IN CHRONIC HEPATITIS B

NK CELLEN EN CHRONISCHE HEPATITIS B INFECTIE

Proefschrift ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam op gezag van de rector magnificus Prof.dr. H.G. Schmidt en volgens besluit van het College voor Promoties.

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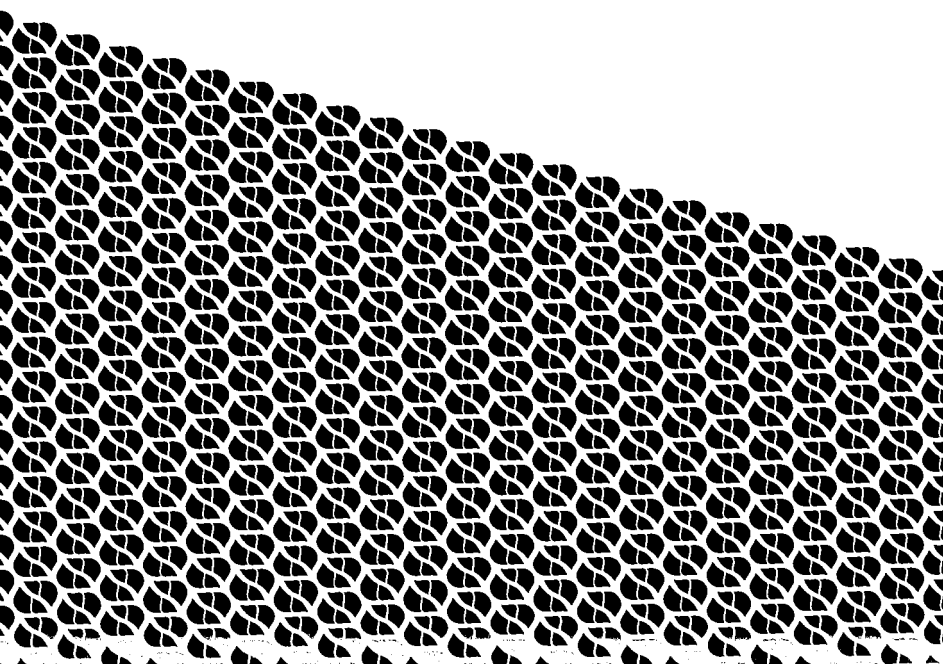
CONTENTS



CHAPTER 1

INTRODUCTION

HEPATITIS B VIRUS (HBV), a member of the family *Hepadnaviridae*, is a 42 nm partially double stranded DNA virus, composed of a 27 nm nucleocapsid core (HBcAg), surrounded by an outer lipoprotein coat (also called envelope) containing the surface antigen (HBsAg). The family of hepadnaviruses are enveloped virions containing 3 to 3.3 kb of relaxed circular, partially duplex DNA and virion-associated DNA-dependent polymerases that can repair the gap in the virion DNA template and have reverse transcriptase activities. Hepatocytes infected in vivo by hepadnaviruses produce an excess of noninfectious viral lipoprotein particles composed of envelope proteins. Persistent infections display pronounced hepatotropism[52].

Despite the presence of an effective prophylactic vaccine, HBV infection is estimated to be one of the most common causes of liver disease worldwide. HBV causes liver disease that varies greatly in severity form person to person[1]. Some subjects control infection efficiently and clear the virus from the bloodstream either without clinically evident liver disease or with an acute hepatitis that can resolve without long-term clinical sequelae. Other patients fail to clear the virus and develop chronic infection. Most chronically infected patients remain largely asymptomatic without life-threatening liver disease but 10-30% develop liver cirrhosis with possible progression to liver cancer[2]. Whereas vertical transmission of HBV from mother to neonate most likely results in chronic hepatitis, approximately 90% of infection during adulthood results in lifelong protective immunity. Outcome of infection and the pathogenesis of liver disease are determined by virus and host factors, which have been difficult to fully elucidate because the host range of HBV is limited to man and chimpanzee whereas HBV is non-cytopathic for the infected hepatocytes by itself[3].

INNATE IMMUNE RESPONSES IN HEPATITIS B VIRUS INFECTION

The incubation period of a viral disease refers to the time between infection and the onset of symptoms, and is believed to represent the phase of active viral replication before an effective host immune response is developed. This incubation period is generally longer (8-20 weeks) after infection with HBV, than the period following infection with other viruses such as HIV (3-10 weeks)[4]. It is known that immune events central to HBV control occur during the incubation phase and before symptomatic disease. A marked reduction in virus levels before the onset of clinically evident disease, and with limited liver injury, have been shown in human and animal infected with hepadnaviruses[5-7].

What are the components of the immune response that may play a major role in this reduction of HBV levels before the onset of symptoms? In general, innate immunity plays a role immediately after infection to limit the spread of the pathogen and initiate efficient development of an adaptive immune response. Innate host response during the early phases of viral infection are mainly characterized by the production of type I interferon (IFN)-α/β cytokines and the activation of natural killer (NK) cells[8]. Production of type I IFNs can be triggered directly by virus replication through cellular mechanisms that detect the presence of viral RNA or DNA[9].

Despite the lack of an *in vitro* cell culture system to sustain infection and replication of HBV, evidence that HBV is a weak inducer of type I IFNs is present in the literature. In animals, large doses of HBV inoculums do not enter an exponential phase of replication until 4-5 weeks[10]. The initial lag phase of HBV replication does not appear to be a consequence of HBV inhibition by elements of innate and adaptive immunity. The activation of IFN-γ, interleukin (IL)-2 and tumour necrosis factor (TNF)-α and intrahepatic recruitment of inflammatory cells is delayed until the logarithmic expansion of HBV in experimental animal models[6, 11-12]. In these models, hardly any cellular genes were activated within the liver during the lag phase of infection, confirming that intrahepatic activation

of innate immunity did not affect initial HBV spread. Interestingly, early IL-6 mediated control of HBV infection at the transcriptional level has been observed limiting activation of the adaptive immune response and preventing death of the HBV-infected hepatocyte[15]. This pattern recognition may be essential for a virus, which infects a new host with only a few virions.

The causes of the delayed appearance of quantifiable levels of HBV proteins and HBV-DNA in the first weeks of infection are not clear and thus we do not know whether this apparent initial vanishing has an impact on the natural history of disease. A characteristic of HBV in relation to early host defence mechanisms resides in the lack of IFN-α and β production. HBV replication can be efficiently limited by α and β IFN[14], but animal studies show that such antiviral cytokines are not triggered by HBV replication[15]. It has been proposed that because HBV replicates within nucleocapsid particle, viral replicative intermediates of single-stranded RNA or viral DNA, generally strong activators of type I IFNs, are protected from cellular recognition[12]. Such early events are difficult to analyse during natural infection in humans. HBV-infected patients are mainly detected after onset of clinical symptoms, which occur well after infection (10-12 weeks). Nevertheless, it is interesting to note that the lack of early symptoms in HBV-infected patients such as fever and malaise, which are characteristic of other human viral infections, constitutes indirect evidence of the defective type I IFN production during the early phase of HBV infection[16].

In animal models with acute HBV infection, robust activation of IFN-γ, TNF-α and many cellular genes linked to an adaptive response was observed after the exponential phase of HBV expansion[6, 15]. Furthermore, rapid drop in virus replication occurs in the presence of intrahepatic IFN-γ production, before the massive recruitment of T cells[6]. Animal models that develop HBV chronicity lack the large IFN-γ and TNF-α production observed in resolved animals[17] and fail to develop an efficient antiviral-specific immune response. In line with these observations, virtually all patients that experience acute hepatitis B resolve the infection, whereas development of chronicity is often associated with absent or mild symptoms of acute hepatitis. Activation of components of

innate immunity, capable of producing large quantities of IFN-γ, could be a factor that determines the subsequent efficient induction of adaptive immunity and ultimately the outcome of HBV infection. The difference in the adaptive immune response to HBV that characterize chronic and resolved patients are heavily influenced by the immunological events occurring during the initial phase of HBV replication.

NATURAL KILLER CELLS; A FRIEND IN ANTIVIRAL WARFARE

NK cells contribute to the innate immune defence against microbial pathogens such as viruses. NK cells use multiple mechanisms to defend against viral infections, and different stimuli can activate these antiviral effects. When engaged, receptors for innate cytokines produced during infection and for ligands on target cells can both induce NK cell cytotoxicity and the production of cytokines.
NK cells sense their environment by using a sophisticated repertoire of receptors that allows them to distinguish between normal cells and cells infected with intracellular pathogens[18]. NK cells are most abundant in blood and liver, where they comprise almost 1/3 of the intrahepatic lymphocyte population[19]. NK cells can be easily identified by flow cytometry by the expression of CD56 in the absence of typical markers of T-cell and B-cell lineages. They can be further divided in two subsets on the basis of intensity of expression of the CD56 molecule. Thus, $CD56^{dim}$ cells represent the majority of circulating NK cells and are considered developmentally mature, whereas $CD56^{bright}$ cells are the minority and thought to be at an earlier stage of maturation[20], although viral infections may occasionally alter these proportions[21]. Moreover, $CD56^{dim}$ cells have been shown to predominantly mediate cytotoxicity, whereas $CD56^{bright}$ cells appear to principally secrete cytokines[22]. Contrary to T and B cells that display sophisticated rearranged antigen-specific receptors, NK cell receptors (NKRs) are in germ-line configuration. NK

effector function is controlled by a complex network of signals, which interact with membrane-expressed inhibitory and activating receptors[25]. The critical role of inhibition in maintaining immunological homeostasis has been emphasized by the 'missing self' hypothesis explaining why healthy cells expressing MHC class I are not killed by NK cells[24]. 'Resting' mature NK cells (i.e. dominant inhibitory signalling) constitutively express transcripts for IFN-γ[25] and they contain pre-formed cytolytic mediators, such as perforin and granzymes, stored in intracellular granules[26].
The exact mechanisms of activation remain the subject of current investigation, but recognition of an "altered self" state is thought to be involved. To control their cytotoxic activity, inhibitory receptors of NK cells recognize MHC class I alleles, which could explain why NK cells kill cells possessing low levels of MHC class I molecules.
Activation allows the recognition of altered self via binding to ligands, which are expressed by stressed cells, effectively functioning as danger signals[18]. These ligands include classical MHC class I molecules, as well as other molecules, such as MHC class I-like stress-induced self ligands (MICA/B). Downregulation of MHC class I or loss of its expression during viral infection lifts the inhibitory signal to NK cells leading to activation and granular exocytosis[27].
Among inhibitory receptors, killer inhibitory receptors (KIRs) have been observed to play a role in the outcome of viral infection, notably HCV[28]. HCV has also devised means to evade detection by NK cells[29], which emphasizes the importance of NK cells in host defence. Members of the C-type lectin-like receptor family, including the NKG2A receptor recognizing HLA-E, are also among the inhibitory receptors. However, the C-type lectin-like receptor NKG2D belongs to the activating receptors and binds to ligands MICA/B that are poorly expressed on healthy cells, but which are upregulated by viral infection ('induced self' recognition)[30]. Other important activating receptors are the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46, which are exclusively expressed by NK cells and for which no cellular ligands have been formally identified.
NK cells may also be activated by the cytokine environment notably type I IFNs or pro-inflammatory cytokines such as IL-15, IL-12 and IL-18, becoming fully functional effector cells that can provide optimal host

defence against viral infections. In many situations, dendritic cells (DC) are probably the main source of the type I IFN and IL-12 that is necessary for NK cell activation[51], and in turn, TNFα and IFN-γ produced by NK cells can affect the maturation and effector functions of DC either directly or in synergy with suboptimal levels of viral signals[52-53]. Immature DC appear susceptible to autologous NK cell mediated cytolysis while mature DC are protected[54]. This bi-directional cross talk between DC and NK cells appears to play an important role in both innate and adaptive immune responses to pathogens. In inflamed tissue, the simulatenous engagement of Toll like receptors (TLR), which are expressed by both cell types results in cell activation and the acquisition of functional properties necessary for controlling, and possibly rapidly eliminating, viruses by innate effector mechanisms. Moreover, NK cells are needed to select the most appropriate DC that display the functional properties suitable for subsequent T cell priming[55].

Thus, cognate cell-cell recognition is not absolutely required for NK cells to participate in a response to a virus-infected cell because NK cells can also be activated as bystander cells simply by exposure to IFNs and cytokines in their environment. For example, culture of NK cells with IL-12 and IL-18 (or other combinations of cytokines with IL-12) is sufficient to initiate secretion of IFN-γ, without requiring any deliberate engagement of the activating receptors expressed by NK cells that detect alterations in the cell surface of the infected cells. In addition, because NK cells express CD16, an activating Fc receptor for IgG, they can theoretically attack virus-infected cells that are coated with IgG, with specificity being contributed by the antibody. Therefore, the relative contribution of and the effector mechanisms involved in an NK-cell response to a given pathogen can vary considerably. Interestingly, despite it is generally believed that NK cells do not need priming, which would confer early protection against intracellular pathogens, it has been suggested that prior exposure to pathogens significantly shorten the time required for activation upon rechallenge[56], casting doubts on the current dogma that NK cells do not have memory. NK cells isolated after infection with any of several different viruses had increased cytolytic activity *in vitro* against cellular targets, and viral infection can result in NK cell proliferation and recruitment to the infected tissues

and organs[37]. In many cases, the increased NK cell-mediated killing was attributed to activation of NK cells by the production of type I IFNs induced in the host by viral infection, because it was known that NK cells could be directly stimulated by exposure to IFN-α and IFN-β[38]. Although type I IFNs are not mitogenic for NK cells, they do induce the production of IL-15[39], which is a potent growth factor for NK cells. In addition, IL-15 is a potent stimulus for cytokine production by NK cells and augments cytotoxicity. Hence, the induction of type I IFNs and IL-15 by viral infection could well account for the presence of activated NK cells in virus-infected hosts[25].

NATURAL KILLER CELLS IN HEPATITIS B INFECTED PATIENTS

There is a bulk of evidence in mostly murine models showing that, besides cytotoxicity, the initial burst of IFN-γ and the subsequent rapid inhibition of HBV may be mediated by NK cells before the advent of HBV-specific CD8 T cells[8, 40-43].

There are very few studies that have looked at human NK cells during acute viral hepatitis. Consistent with the evidence for their role in the early phase of infection, a high number of circulating NK was found in the early period of the incubation phase of patients who developed acute hepatitis B[5, 44]. The number of NK cells declined in association with a drop in the level of HBV-DNA suggesting a role in viral containment. However, their activation and effector function was suppressed as viral load increased and only peaked once viremia had resolved[16]. Moreover, it is observed that innate cytokines such as IFN-α, IL-15 and IFN-λ1 remained barely detectable throughout the incubation period in acute hepatitis B, and NK cell production of IFN-γ was also defective particularly at peak viremia[16]. This was ascribed to a burst of the immunosuppressive IL-10 production in the early stages of acute HBV infection and would be compatible with the deficient type I IFN response documented in the chimpanzee model of acute HBV infection[15]. Induction of IL-10 was not detected in patients

with an asymptomatic mild course of acute HBV, in line with the lack of attenuation of NK cell activity at peak viremia in patients with this clinical pattern of the disease[44]. NK cells may also contribute to liver damage in acute HBV infection, as hepatocyte death through NK cell mediated apoptosis coinciding with peak liver inflammation was observed[45].
The reports on quantification of the number of circulating NK cells, examining their phenotype and correlating those parameters to NK cell function in patients with chronic HBV infection are scarse and inconsistent[45-59]. The reasons for such discrepancies are not immediately apparent, although they may be due, in part, to clinical, genetic, biochemical and virological heterogeneity of patients.

THERAPY FOR CHB

First-line treatment options for chronic hepatitis B (CHB) consist of nucleos(t)ide analogues with a high barrier to resistance (entecavir and tenofovir) or the immunomodulatory agent peginterferon (PEG-IFN). The optimal choice for individual patients remains controversial[51]. Long-lasting, treatment-maintained suppression of hepatitis B virus (HBV) DNA without resistance is achievable in most patients by entecavir or tenofovir. A sustained off-treatment response is, however, unlikely and long-term therapy must be anticipated. PEG-IFN offers a higher rate of sustained response in a subgroup of patients, but is frequently complicated by side effects.
Optimalization of treatment efficacy of current therapeutic agents may be achieved in challenging the above paradigms. Furthermore, chronic hepatitis B may be regarded as an entity with various phases necessitating to adapt therapy considering the distinct characteristics of each different phase of chronic infection[2]. Understanding the immune response in blood and liver during chronic HBV infection and how it is altered upon therapy by reducing viral load and HBV proteins may facilitate a rational approach to an individualized treatment most likely to achieve viral clearance.

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OUTLINE OF THE THESIS

From the very early start of the project, the first paper I encountered carried the auspicious title "NK cells in chronic hepatitis B infection; an underinvestigated innate immune response" by Chen et al. in the Journal of Viral Hepatitis 2005. Until then and in contrast to acute HBV infection, no in-depth studies had been conducted on the premise that NK cell activity was confined to early acute infection, while an efficient cytotoxic T cell response was thought to be instrumental in viral clearance.

It becomes increasingly clear that NK cells play a central role in the crosstalk between innate and adaptive immunity. The **GENERAL HYPOTHESIS** underlying the thesis is that an impaired innate immune response, the NK cell response in specific, determines the ineffective adaptive immune response to HBV leading to viral persistence.

In the continuous presence of the virus and its proteins, it is not feasible to determine whether the studied NK cell response is the cause or consequence of chronic HBV infection. The **AIM OF THE THESIS** is thus to investigate, through a translational approach, the role of NK cells in chronic hepatitis B. For this, we describe the phenotype and function of both blood and liver NK cells during different phases of chronic HBV infection and after antiviral therapy, limiting the influence of viral DNA and proteins.

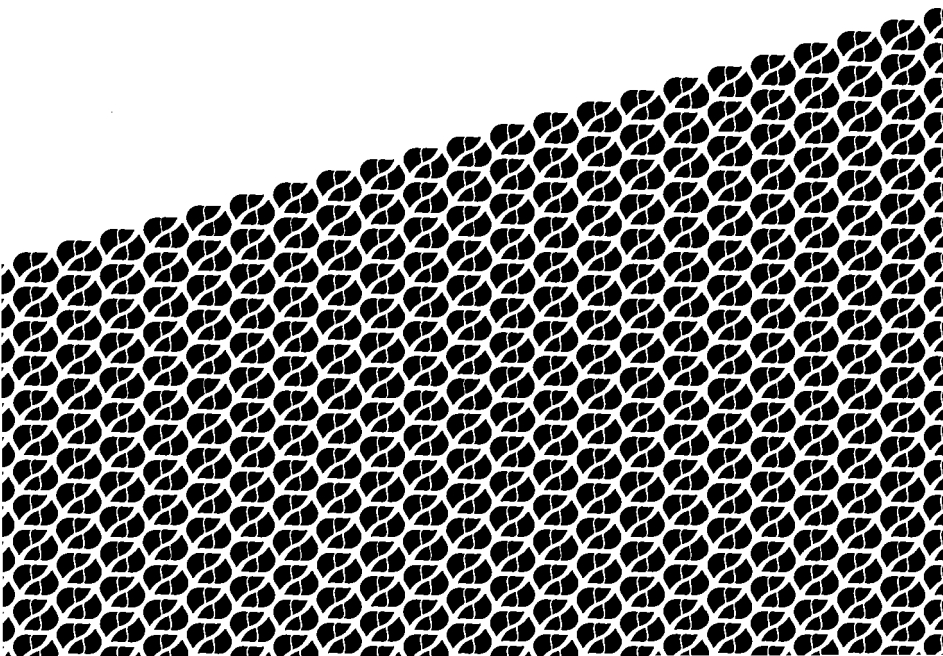
CHAPTER 2 describes the results of a cross-sectional study on blood NK cells showing phenotypical and functional differences between healthy controls and chronic HBV patients. Furthermore, chronic HBV patients were treated with the nucleoside analogue entecavir to study the effect of antiviral therapy on blood NK cells in these patients.

The results included in **CHAPTER 3** and **CHAPTER 4** address the hypothesis that NK cell function is dependent on the interaction with the other key cellular components of the innate immune response namely the dendritic cells (DCs). In chapter 3, an *in vitro* study on the interaction of blood NK cells with plasmacytoid DCs, principal producers of type I IFNs, in the presence of HepG2.15 cell-line derived HBV virus is described. Chapter 4 reports on an *ex vivo* study on the interaction between blood

NK cells and myeloid DCs and the effect of nucleos(t)ide analogue therapy (entecavir and tenofovir) on this interaction between both cells directly isolated from chronic HBV infected patients.

CHAPTER 5 and **CHAPTER 6** concern the studies performed on intrahepatic NK cells of chronic HBV patients. NK cells present in liver biopsies of both HBeAg positive and negative chronic hepatitis B patients were evaluated for activation status and function. Furthermore, we tested for the first time the intrahepatic NK cell response during antiviral therapy with tenofovir by repetitive sampling through fine needle aspiration biopsies.

Finally, in **CHAPTER 7** the findings in this thesis will be put into perspective with current knowledge and future directions will be discussed.



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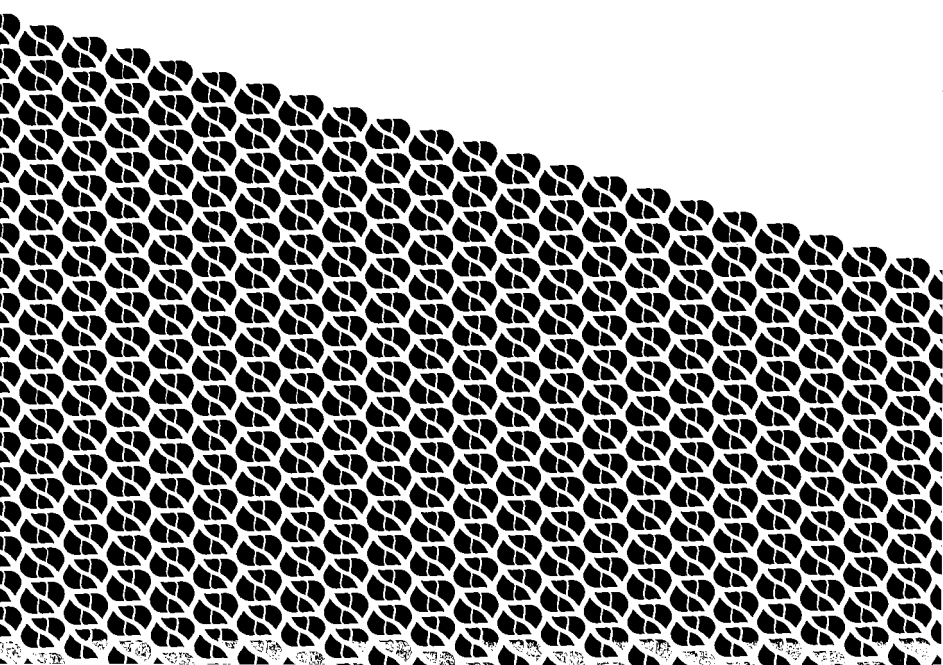


CHAPTER 2

VIRAL LOAD REDUCTION IMPROVES ACTIVATION AND FUNCTION OF NATURAL KILLER CELLS IN PATIENTS WITH CHRONIC HEPATITIS B

ABSTRACT

Natural killer (NK) cells play a major role in anti-viral immunity as first line defense and regulation of virus-specific T cell responses.

OBJECTIVE: To investigate phenotype and function of NK cells in patients with chronic hepatitis B virus (HBV) infection and the effect of anti-viral therapy.

METHODS: Peripheral blood NK cells from 40 chronic HBV patients were compared to NK cells of 25 healthy controls. The effect of entecavir-induced viral load reduction on NK cell phenotype and function were investigated in 15 chronic HBV patients.

RESULTS: NK cell numbers and subset distribution did not differ. Concerning their function, the cytotoxic capacity was retained, whereas NK cell activation and subsequent IFNγ and TNFα production, especially of the $CD56^{dim}$ subset, were strongly hampered in chronic HBV. This functional dichotomy was paralleled by an altered activation state, elevated expression of NKG2A and downregulated expression of CD16 and NKp30, which correlated with serum HBV-DNA load. Anti-viral therapy partially restored NK cell phenotype, as shown by NKG2A downregulation. Moreover, viral replication inhibition improved IFNγ production as a result of an increased ability of $CD56^{dim}$ NK cells to become activated *de novo*. This improved NK cell activation and function correlated with therapy-induced reduction in serum ALT levels, but not HBV-DNA load.

CONCLUSIONS: The specific defect in $CD56^{dim}$ NK cell activation and the reduced capacity to produce anti-viral and Th1-skewing cytokines may play a role in HBV persistence. Restoration of this NK cell cytokine producing capacity as achieved by viral load reduction could therefore contribute to definite clearance of the virus.

INTRODUCTION

Chronic hepatitis B is the result of an inadequate immune response towards the virus[6,19]. Current treatment strategies should be improved to achieve effective anti-HBV immunity in all patients. The development of an effective anti-HBV therapy requires insight in the mechanisms of HBV persistence. It is known that the lack of a coordinated and efficient T cell response against HBV is underlying the viral persistence[5,19], but the mechanism behind this failure has not been elucidated.
Natural killer (NK) cells are innate immune cells that are crucial in the defense against viral infections[4,32]. Human NK cells can be divided into two subsets based on cell-surface density expression of CD56[11,38]. Despite the absence of antigen-specific receptors at their surface, NK cells can selectively eliminate virus-infected cells. A dynamic and precisely coordinated balance between activating and inhibitory receptors governs NK cell activation programs[24,28]. Also a pro-inflammatory cytokine milieu can skew NK cells to become functionally active[4,15].
NK cells mediate their anti-viral effects through at least three different mechanisms[36]: (1) release of cytolytic granules for lysis of infected cells, (2) induction of target cell apoptosis through crosslinking of cell surface death receptors, and (3) release of cytokines. However not absolute, cytotoxicity is mainly exerted by the $CD56^{dim}$ subset, whereas the $CD56^{bright}$ subset is specialized in the release of cytokines[12]. In addition to the direct anti-viral effect, NK cell-derived cytokines, notably IFNγ, are important for skewing T cell responses towards Th1 and subsequent cytotoxic T cell responses[26].
Only limited information has been published on the role of NK cells in HBV infection. In acute HBV, an early rise in circulating NK cells has been documented, suggesting their contribution to the initial viral containment[17,29,37]. In the context of persistent HBV infection, NK cell studies mainly focussed on NK cell-induced tissue injury[9,14]. However, only little is known about the quality of the anti-viral functions of NK cells during chronic HBV, and the effect of anti-viral therapy on these cells has not been reported. By analysing the function of peripheral blood NK cells of chronic HBV patients prior to and during anti-viral therapy, we provide

insight in the anti-viral function of NK cells during chronic HBV infection and the effect of viral load reduction on NK cell phenotype and function. The data show that the cytotoxic capacity is retained, whereas NK cell activation and IFNγ production are strongly hampered in chronic HBV, which may contribute to viral persistence. Anti-viral therapy resulted not only in a partially enhanced activation state of NK cells *in vivo*, but also in improved production of IFNγ as result of an increased susceptibility for *de novo* activation *ex vivo*. These findings may contribute to the development of novel therapeutic approaches to clear the viral infection.

PATIENTS AND METHODS

PATIENTS AND HEALTHY SUBJECTS. Peripheral heparinized blood samples were obtained from 40 patients with chronic hepatitis B for cross-sectional analysis and from 15 other patients for longitudinal analysis during anti-viral therapy (TABLE 1). Blood samples of the latter group were obtained at baseline (t=0) and after 6 months (t=6) of anti-viral therapy. All patients were negative for antibodies against hepatitis C, hepatitis D and human immunodeficiency virus. Patients were treated with the nucleoside analogue entecavir (0.5 mg o.i.d.) and all of them met the most recent EASL-guideline criteria for treatment of chronic HBV[15]. Patients receiving anti-viral therapy within 6 months prior to treatment with entecavir were excluded. An age and sex-matched control group comprised 25 healthy subjects. The study was approved by the local ethics committee, and all patients and controls in the study gave informed consent before blood donation.

PERIPHERAL BLOOD NK CELL ENUMERATION. Whole blood samples were treated with ammonium chloride solution to lyse erythrocytes. To determine the percentage of NK cells, incubation with a cocktail of phycoerythrin (PE)-conjugated CD56 (MY31, BD Bioscience, Belgium), Pacific Blue-conjugated CD3 (UCHT1, Biolegend, USA) and AmCyan-

TABLE 1. CHARACTERISTICS OF STUDY POPULATION

	Healthy controls (n=25)	Chronic hepatitis B (n=40)	Entecavir-group (baseline, n=15)
Age, years [mean ± SEM]	36.9 ± 1.6	38.1 ± 1.6	43.1 ± 3.3
Sex Female/male (%)	11/14 (44/56)	13/27 (33/67)	2/13 (26/73)
ALT, IU/mL [median ± IQ range]	n.a.	60 (34-145)	75 (49-101)
HBV-DNA mean log^{10} cps/mL ± SEM	n.a.	6.5 ± 0.3	7.7 ± 0.6
HBeAg Positive/negative (%)	n.a.	11/29 (25/75)	5/10 (33/67)
HBV genotype Genotype A/B/C/D/E	n.a.	10/4/4/20/2	5/1/2/6/1

Abbreviations: SEM; standard error of mean, IQ; interquartile, n.a.; not applicable

conjugated CD45 (2D1, BD Bioscience, Belgium) was done for 15 minutes. Stained cells were analysed using a multi-colour flow cytometer (FACS Canto II) and Diva software (both BD Bioscience, Belgium).

EXPRESSION OF INTRACELLULAR AND CELL SURFACE MOLECULES BY FLOW CYTOMETRY. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Isopaque (GE Healthcare, Finland) gradient centrifugation. For phenotypic analysis, incubation with a cocktail of fluorescein isothiocyanate (FITC), PE, peridinin-chlorophyll-protein complex (PerCP) and allophycocyanin (APC)-conjugated monoclonal antibodies (mAbs) was performed. The mAbs directed against the following molecules were used: CD3 (UCHT1), HLA-DR (L243), CD69 (L78) from BD Bioscience, Belgium; perforin (ΔG9; BD Pharmingen, USA); CD56 (N901), NKG2D (ON72), CD158a,h (EB6B), CD158b1,2 (6L-183), CD11a (121/7), CD85j (HP-F1), NKp30 (Z25), NKG2A (Z199), CD94 (HP3B1) from Beckman Coulter, USA; CD16 (LNK16, Exbio Nuclilab, Czech Republic), CD11c (KB90, Dako, Denmark) and granzyme B (GB11, Sanquin, The Netherlands). As controls, cells were stained with corresponding isotype-matched control antibodies. For perforin and granzyme B staining, PBMC were fixed in 2% formaldehyde. After washing, PBMC were permeabilised with 0.5% saponin and then cells were incubated with mAbs. Stained cells were analysed using a 4-color flow cytometer (FACS Calibur) and

CellQuest software (both BD Bioscience, Belgium). Mean fluorescence intensity (MFI) and/or percent positive cells were determined.

CYTOTOXICITY ASSAYS. To test natural cytotoxicity, cryopreserved NK cells were tested against the MHC I-lacking K562 cell line and Daudi cell line in a standard 4h-^{51}chromium (^{51}Cr)- release (cytotoxicity) assay. The NK cell resistant cell line Daudi was also used to test lymphokine (IL-2) activated killing (LAK) capacity. Upon recovery, PBMC were cultured for 18h in RPMI 1640 containing 25 mmol/L HEPES, 2 mmol/L L-glutamine, 50 µg/mL streptomycin (all Lonza, Belgium), 50 U/mL penicillin (Invitrogen, USA), and 10% fetal calf serum (Hyclone, The Netherlands) in the absence or presence of 800 U/mL recombinant human IL-2 (Strathman, Germany), to measure natural and IL-2-induced cytotoxicity, respectively. PBMC were then incubated with 2500 target cells labeled with 100µCi ^{51}Cr (Na_2CrO_4) (MP Biomedicals, USA) at different effector-target ratios in roundbottom 96-well plates. Maximum ^{51}Cr release was determined by incubating target cells in 10% Triton X-100. For spontaneous release, targets were incubated without effectors in medium alone. ^{51}Cr release was measured after 4h in an automatic gamma counter (Perkin Elmer, 2480 Wizard, The Netherlands). All samples were performed in duplicate or triplicate. Specific lysis (%) was calculated as: (measured ^{51}Cr release - mean spontaneous ^{51}Cr release) / (maximal ^{51}Cr release - mean spontaneous ^{51}Cr release) * 100%

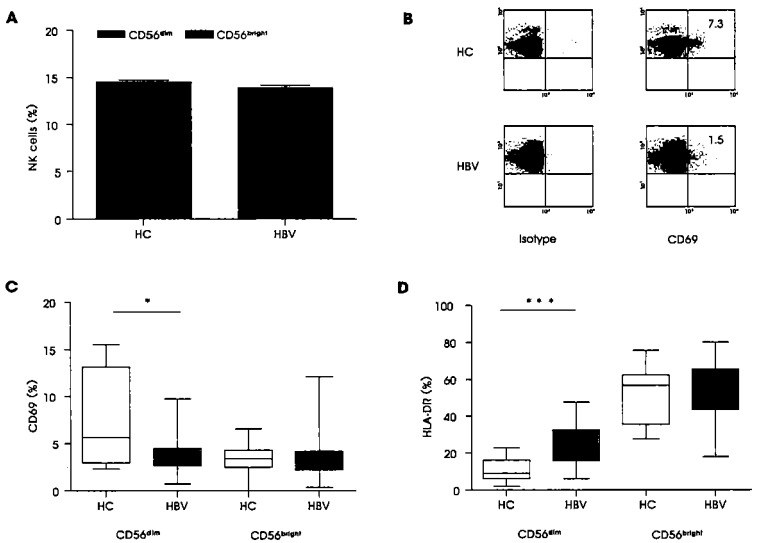
CD107 DEGRANULATION ASSAY. To measure degranulation of NK cells in response to stimulation with target cells, 25x10^3 PBMC with or without IL-2 pre-incubation were cultured with target cells. PE-conjugated mAbs to the granular membrane protein LAMP-1 (CD107a, H4A3, BD Pharmingen, USA) or isotype were added to PBMC prior to stimulation with target cells (K562/Daudi) in a 10:1 effector-target ratio. The cultures were incubated for 1h at 37°C in a humidified 5%CO_2 incubator, followed by an additional 3h in the presence of the secretion inhibitor brefeldin A (10 µg/mL, Sigma). Control samples were incubated without target cells to detect spontaneous degranulation. Thereafter, samples were stained on ice with PerCP-conjugated CD3 and APC-conjugated CD56 mAbs, followed by FACS analysis.

CYTOKINE PRODUCTION. Cytokine production by NK cells present in PBMC or isolated NK cell ($0.5x10^6$ cells/mL) cultures were determined by stimulation with 100 ng/mL IL-18 (MBL International Corporation, Japan) plus 10 ng/mL IL-12 (Miltenyi Biotec, Germany) for 24h in 24-well plates. NK cells from PBMC of a randomly selected group of patients and healthy controls were isolated by negative selection (purity and viability >95%) with an NK cell isolation kit (Miltenyi Biotec, Germany). After 21h stimulation, 10 µg/mL brefeldin A was added. In a selected group of patients and controls, PBMC were also stimulated with 50 ng/mL PMA and 2 µg/mL ionomycin (both from Sigma Aldrich, USA) for 4h. After 1h stimulation, Brefeldin was added. Non-stimulated cells were treated similarly and used as controls. PBMC or isolated NK cells were fixed in 2% formaldehyde. After washing, cells were permeabilised with 0.5% saponin after which incubation with FITC-conjugated antibodies against IFNγ (B27, BD Pharmingen, USA) or APC-conjugated anti-TNFα (MP9-20A4, Caltag, UK) was performed. Cells were also stained with monoclonal antibodies specific for surface CD3, CD56, CD69 and detected by FACS. Results are expressed as percentage of cytokine producing cells within the NK cell population. Levels of IFNγ and TNFα present in culture supernatants were determined by standard enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (eBioscience).

STATISTICAL ANALYSIS. Data are expressed as mean±SEM or presented in box-and-whisker plots, which demonstrate the median, the lower and upper quartiles, and the minimum and maximum values in the distribution, unless indicated otherwise. Data were analysed with Prism 5.0 (GraphPad software, USA) using the Mann-Whitney U test to compare variables between 2 independent groups, the Wilcoxon matched pairs test between paired variables and the Spearman's rank correlation coefficient test for non-parametric correlations. In all analyses, a two-tailed *P*-value of less than *.05* (confidence interval 95%) was considered statistically significant.

ALTERED FREQUENCIES OF ACTIVATED CD56^{dim} NK CELLS IN CHRONIC HBV PATIENTS. In patients as well as controls, the peripheral blood lymphocyte population contained about 14% NK cells, with equal distribution in both subsets (FIGURE 1A). We determined the *in vivo* expression of the early activation marker CD69 and the late activation marker HLA-DR on NK cells. In chronic

FIGURE 1. FREQUENCY OF EARLY ACTIVATED CD56^{dim} NK CELLS IS DECREASED IN PATIENTS WITH CHRONIC HBV INFECTION



ABC: Fresh PBMC obtained from 15 healthy controls (HC) and 30 sex and age-matched chronic HBV patients (HBV) (mean±SEM HBV-DNA ^{10}log 6.2±0.4 cps/mL ALT 104±21 IU/mL 22 e-negative Ag) were investigated for the frequency and subset distribution of CD56+CD3- NK cells within the lymphocyte gate (A) and their positivity for the early activation marker CD69 (C) and late activation marker HLA-DR (D) by FACS analysis. Representive FACS plots of CD3-CD56+ cells stained for CD69 are shown (B). NK cell frequencies are shown as mean±SEM (A), the percentage of NK cells positive for activation markers are presented in box-and-whisker plots (BC). * *P<.05, ***P<.001*

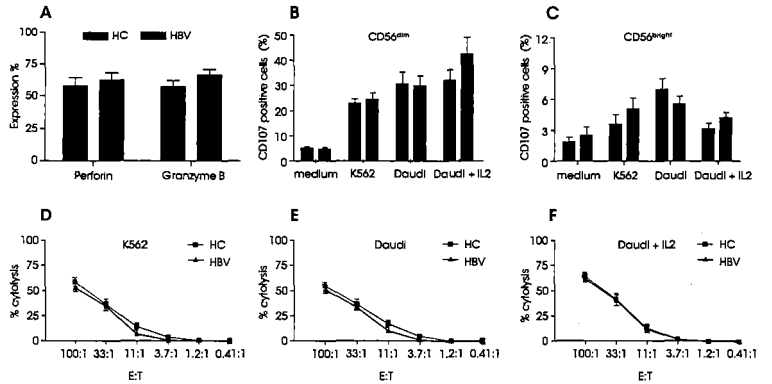
HBV, CD56^{dim} NK cells less frequently expressed CD69 (FIGURE 1BC) but more HLA-DR than controls (FIGURE 1D). Within the CD56^{bright} NK cells HLA-DR expressing cells were not increased, but like the CD56^{dim} cells the percentage of CD69-positive cells tended to be diminished. Considering the total NK cell pool, viral load was inversely correlated with levels of CD69 positivity ($r=-.42$, $P<.05$), but not with HLA-DR expression. Neither HLA-DR nor CD69 expression correlated with serum ALT levels or eAg-status. These data indicate that in chronic HBV infection NK cells, especially the CD56^{dim} subset, display a terminally activated status, i.e. low CD69 and high HLA-DR expression.

NK CELL DEGRANULATION AND CYTOTOXICITY IS INTACT IN CHRONIC HBV PATIENTS. NK cells are capable of direct cytolysis, which is dependent on release of granules containing perforins and granzymes[12]. To assess the cytotoxic capacity of NK cells in chronic HBV patients, PBMC of HBV patients and controls were stained for intracellular perforin and granzyme B. Neither the total percentage of perforin- or granzyme B-positive NK cells nor the expression level differed between the groups (FIGURE 2A, data not shown).

Degranulation and subsequent cytolysis of target cells were determined by assessing CD107a expression and direct cytotoxicity, respectively, upon co-culture with K562 or Daudi cells. Co-culture with these cell lines induced degranulation in CD56^{dim} and to a lesser extent in CD56^{bright} NK cells that did not differ between NK cells derived from patients and healthy controls (FIGURE 2BC). Parallel to degranulation, the killing capacity of NK cells was similar between groups for natural cytotoxicity as well as LAK activity (FIGURE 2DEF). Thus, NK cells of chronic HBV patients do not differ in their cytotoxic capacity from NK cells circulating in healthy controls.

NK CELLS OF CHRONIC HBV PATIENTS DISPLAY AN IMPAIRED CAPACITY TO PRODUCE IFNγ. Next to their cytotoxic capacity, cytokine production by NK cells is of major importance in antiviral immunity. Although CD56^{bright} NK cells were originally considered the cytokine producing NK cell subset, it is now clear that also CD56^{dim} NK cells significantly contribute to cytokine production. Indeed, stimulation

FIGURE 2. INTACT NK CELL CYTOTOXICITY IN PATIENTS WITH CHRONIC HBV INFECTION



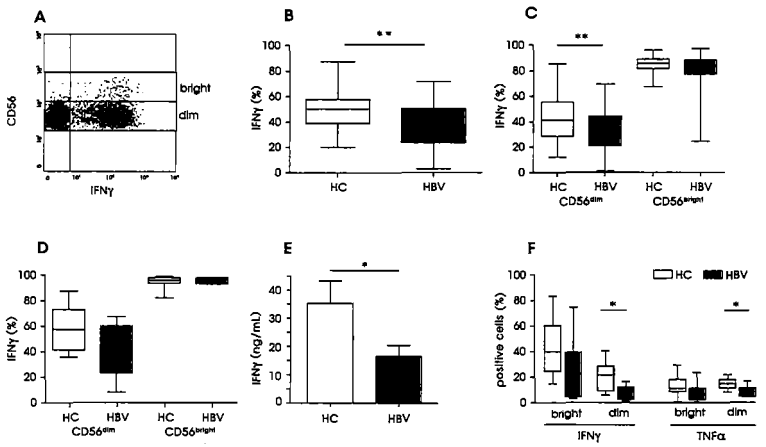
A: Intracellular granzyme B and perforin expression were determined in NK cells present in fresh PBMC of 15 healthy controls (HC) and 15 sex and age-matched chronic HBV patients (HBV) (mean±SEM HBV-DNA ^{10}log 6.5±0.6 cps/mL, ALT 84±20 IU/mL, 9 e-negative Ag) by FACS analysis. Data shown represent mean±SEM percentage of perforin or granzyme B expressing cells within the total NK cell population.

BC: CD107 expression on CD56^{dim} (B) and CD56^{bright} (C) NK cells within fresh PBMC, that were either left untreated or stimulated with IL-2, after co-culture with K562 or Daudi cells. Data shown represent mean±SEM percentage of CD107 expressing NK cells within the different subsets of 15 healthy controls (HC) and 15 chronic HBV patients (HBV).

DEF: Natural NK cell cytolysis against K562 (D) or Daudi cells (E) and IL-2 induced killing of Daudi cells (F) were determined in ^{51}Cr release assays. Data represent the mean±SEM percentage cytolysis by PBMC derived from 15 healthy controls (HC) and 15 chronic HBV patients (HBV) in different effector:target ratios.

of PBMC with IL-12 and IL-18 resulted in IFNγ production by almost all CD56^{bright} NK cells, but overall a large proportion of the IFNγ producing cells belong to the CD56^{dim} subset (FIGURE 3A). Intracellular TNFα could not be detected after 24h stimulation with IL-12/18 (data not shown). After 24h incubation of PBMC in the presence of IL-12 and IL-18, patients showed 1.4-fold less IFNγ producing NK cells than healthy controls (FIGURE 3B): the CD56^{dim} subset produced more than 30% less

FIGURE 3. IMPAIRED NK CELL CYTOKINE PRODUCTION IN PATIENTS WITH CHRONIC HBV INFECTION



A: Fresh PBMC were stimulated with IL-12 and IL-18 as described in Materials and Methods. After 24h, IFNγ production was determined by flow cytometry by gating on CD3-CD56+ NK cells. Shown is a representative dot plot of intracellular IFNγ staining in NK cells with subset indication.

B C: Fresh PBMC were stimulated with IL-12 and IL-18 for 24h. Intracellular IFNγ present in NK cells was determined in 25 healthy controls (HC) and sex and age-matched 40 chronic HBV patients (HBV) (see TABLE 1). The IFNγ expressing cells within the total NK cell population (B) and subsets (C) are presented in box-and-whisker plots. ***P<.01*

D: Intracellular IFNγ was determined in IL-12/18-stimulated purified fresh NK cells of 10 healthy controls (HC) and 10 sex and age-matched chronic HBV patients (HBV) (mean±SEM HBV-DNA ^{10}log 6.3±0.7 cps/mL, ALT 70±22 IU/mL, 7 e-negative Ag) by FACS analysis. The IFNγ expressing cells within the NK cell subsets are presented in box-and-whisker plots..

E: Supernatants of IL-12/18 stimulated purified fresh NK cells derived from 10 healthy controls (HC) and 10 chronic HBV patients (HBV) were evaluated for IFNγ content by ELISA. Data demonstrate mean±SEM IFNγ production. **P<.05*

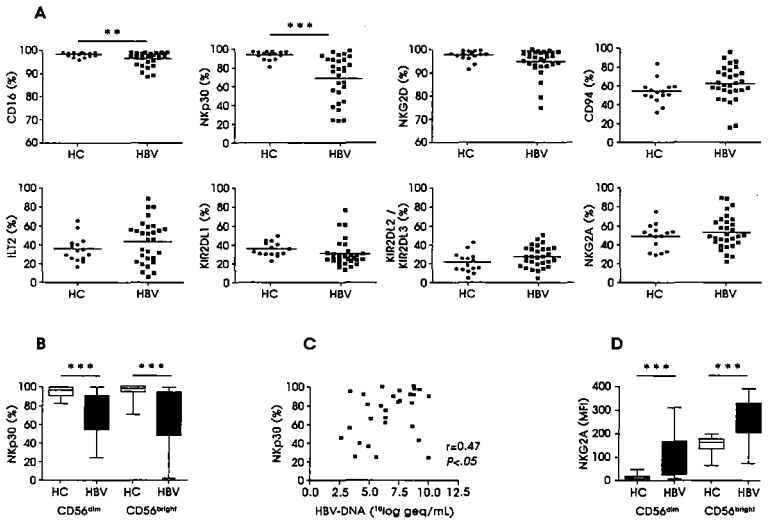
F: Fresh PBMC were stimulated with PMA and ionomycin for 4h. Intracellular IFNγ and TN Fα present in NK cells was determined in 10 healthy controls (HC) and 10 chronic HBV patients (HBV). The cytokine expressing cells within the NK cell subsets are presented in box-and-whisker plots. **P<.05*

IFNγ, whereas CD56^{bright} NK cells expressed 6% less IFNγ compared to healthy controls (FIGURE 3C). Of note, the level of IFNγ expression within the positive cells did not differ between healthy controls and patients (data not shown). The reduced capacity of both total NK cells and NK cell subsets to produce IFNγ did not correlate with viral load, eAg-status or ALT (data not shown). In a smaller group of patients and controls also purified NK cells, especially the CD56^{dim} NK cell subset, of patients tended to a diminished capacity to produce IFNγ as demonstrated by intracellular cytokine detection (FIGURE 3D). Determination of total IFNγ production in these purified NK cell cultures by ELISA revealed that NK cells derived from patients are significantly less capable to produce IFNγ (FIGURE 3E).

Interestingly, this impaired capacity to produce cytokines was not restricted to IFNγ production and IL-12/18 stimulation. PMA/ionomycin induced both IFNγ and TNFα production in NK cells. PMA/ionomycin stimulated cells derived from patients resulted in a 1.7-fold decreased IFNγ and TNFα production by CD56^{dim} NK cells compared to healthy controls (FIGURE 3F). In conclusion, NK cells of HBV patients display functional dichotomy i.e. an intact cytotoxicity, but impaired cytokine production irrespective of the stimulus used.

DIFFERENT CD16, NKP30 AND NKG2A EXPRESSION ON NK CELLS OF CHRONIC HBV PATIENTS. Next, we investigated whether this impaired capacity to produce cytokines was paralleled by an altered expression of activating and inhibitory NK cell receptors. The frequency of CD16 (FcγRIII) expressing NK cells was significantly reduced in patients (FIGURE 4A), whereas the frequencies of NK cells expressing NKG2D, KIR2DL1/2/3, ILT2, NKG2A, and the co-receptor CD94 were not different between the groups. Most pronounced differences were observed for the frequency of NKp30 positive NK cells (FIGURE 4A); the frequency of NKp30^{+} NK cells was almost 20% reduced in both NK cell subsets of patients and correlated with serum viral load (FIGURE 4BC), but not serum ALT levels and eAg-status (data not shown). In contrast to the other molecules described above, which did not differ in the level of expression on the cells positive for the molecules indicated, the expression level of the

FIGURE 4. EXPRESSION OF ACTIVATING AND INHIBITORY RECEPTORS ON NK CELLS IN PATIENTS WITH CHRONIC HBV INFECTION



A: The frequency of activating receptors CD16, NKp30, NKG2D and the NKG-coreceptor CD94 and inhibitory receptors ILT2, KIR2DL1/2/3 and NKG2A on NK cells in fresh PBMC of 15 healthy controls (HC) and 30 sex and age-matched chronic HBV patients (HBV) (mean±SEM HBV-DNA ^{10}log 6.2±0.4 cps/mL, ALT 104±21 IU/mL, 22 e-negative Ag) were determined by FACS analysis. Data show the individual and the mean percentage of expressing cells within the total NK cell population. ***P<.01, ***P<.001*

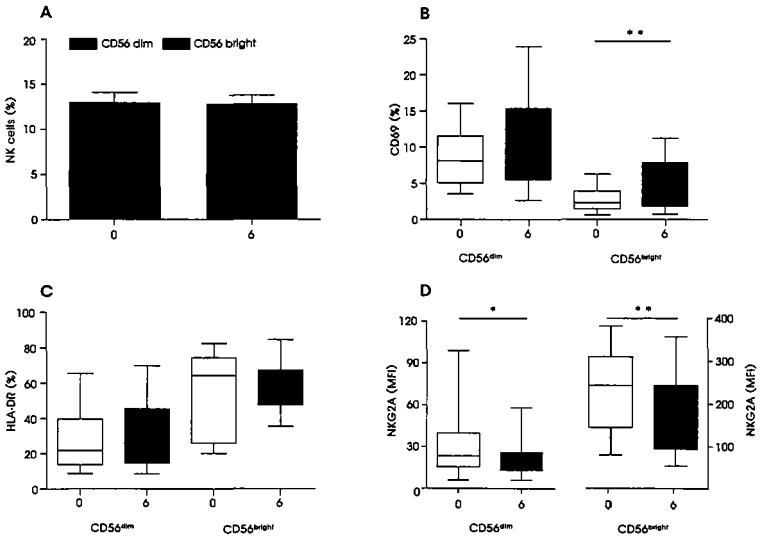
B C: The frequency of NKp30 on both subsets of NK cells within fresh PBMC of 15 healthy controls (HC) and 30 patients with chronic HBV infection (HBV) were determined by FACS analysis. The percentage of NKp30 positive cells within the NK cell subsets are presented in box-and-whisker plots (B) and for the HBV patients related to serum HBV DNA load by determining Spearman's rank correlation coefficient (C) ****P<.001*

D: The expression levels of NKG2A, expressed in mean fluorescence intensity (MFI), on NK cells positive for NKG2A in fresh PBMC of 15 healthy controls (HC) and 30 patients with chronic HBV infection (HBV) were determined by FACS analysis. The expression levels of NKG2A on both NK cell subsets in HC and HBV are presented in box-and-whisker plots (C). ****P<0.001*

inhibitory receptor NKG2A, of which about 50% of NK cells were positive, was two to sixfold higher in both subsets compared to controls (FIGURE 4D, SUPPL. FIGURE 1). However, neither the frequency of NKG2A positive cells nor the expression level of NKG2A correlated with serum viral load, serum ALT levels or eAg-status (data not shown).
Thus, next to impaired cytokine production and altered activation status, NK cells circulating in patients with chronic HBV also display an altered receptor expression that partially correlates with their serum HBV load.

EFFECTIVE VIRAL LOAD REDUCTION UPON ENTECAVIR TREATMENT RESULTS IN INCREASED FREQUENCIES OF ACTIVATED NK CELLS. The effect of viral load reduction on NK cell phenotype and the capacity to produce anti-viral cytokines was examined in chronic HBV patients treated with entecavir, which is a nucleoside analogue that inhibits viral replication without direct effects on the immune system[5]. All patients responded to therapy resulting in a mean $4\log^{10}$ HBV-DNA reduction and mean 2.3-fold lowering of ALT after 6 months of treatment (data not shown). No HBe-seroconversion was achieved within this period.
Viral load reduction neither changed total NK cell numbers nor subset distribution (FIGURE 5A). Considering activation status, the frequency of CD69 expressing $CD56^{bright}$ NK cells was almost doubled upon viral load reduction which indicated elevated numbers of activated cells within this NK cell subset (FIGURE 5B). The individual change in viral load significantly correlated with the change in CD69 positivity on $CD56^{bright}$ NK cells ($r=.58$, $P=.02$), but not on $CD56^{dim}$ NK cells. Also HLA-DR and additional activation markers CD11a and CD11c were determined, but were not differently expressed after viral load reduction (FIGURE 5C and data not shown).
The expression of CD16, KIRs, ILT2, NKG2D, NKp30 on NK cells were also determined on total NK cells and subsets but remained unchanged (data not shown). Viral load reduction had also no effect on the frequency of NK cells positive for NKG2A. However, the level of expression of NKG2A on $CD56^{dim}$ and $CD56^{bright}$ NK cells was significantly reduced to 30% and 85% of baseline expression respectively (FIGURE 5D). Although not significant, percentage of CD94 expressing NK cells tended to be higher in chronic HBV patients (FIGURE 4A). Detailed analysis of CD94

FIGURE 5. VIRAL LOAD REDUCTION RESULTS IN INCREASE OF ACTIVATED CD56^{bright} NK CELLS AND DECREASE OF NKG2A EXPRESSION IN PATIENTS WITH CHRONIC HBV INFECTION

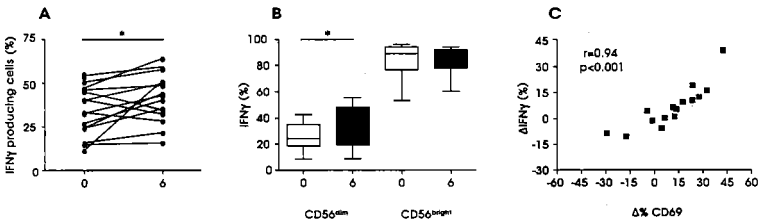


Frozen PBMC were thawed and simultaneously evaluated for the subset distribution of NK cells (A), the frequency of CD69 and HLA-DR expressing NK cells within the NK cell subsets (BC) and the expression level of NKG2A on the cells positive for NKG2A (D) at baseline (0) and at 6 months of therapy (6). Data are presented as mean±SEM (A) or are shown in box-and-whisker plots (BCD). *$P<.05$, **$P<.01$

during antiviral treatment showed that CD56^{dim} NK cells were significantly less expressing CD94, resulting in similar levels as in controls (data not shown). Altered expression of NKG2A and CD94 on both CD56^{dim} as CD56^{bright} NK cells was not correlated with changes in viral load, serum ALT levels or eAg-status (data not shown).

Overall, these data showed that viral load reduction in patients with chronic HBV infection increased the recently activated CD56^{bright} NK cells paralleled by a downregulated NKG2A expression on this subset *in vivo*. The decreased activation of circulating CD56^{dim} NK cells was not significantly improved by treatment despite downregulation of NKG2A on these cells.

FIGURE 6. VIRAL LOAD REDUCTION IMPROVES IFNγ PRODUCTION AND *DE NOVO* ACTIVATION OF CD56^{dim} NK CELLS IN PATIENTS WITH CHRONIC HBV INFECTION



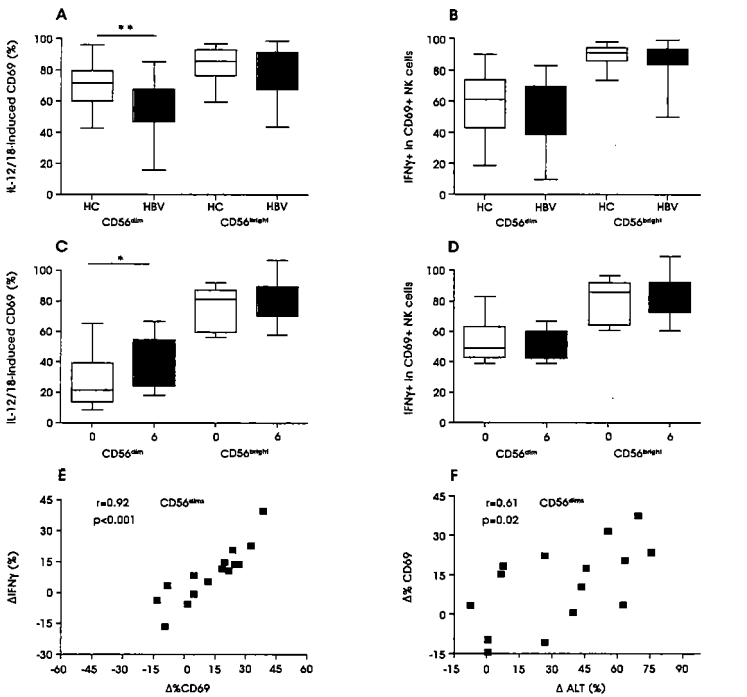
A B: Intracellular IFNγ upon 24h IL-12/18 stimulation was simultaneously determined in total NK cells (A) and NK cell subsets (B) from cryopreserved PBMC at baseline (0) and at 6 months of therapy (6) . Data shown represent the percentage of IFNγ expressing cells in each individual at both timepoints within the total NK cell population (A) and in NK cell subsets as shown in box-and-whisker plots (B). **P<.05*

C: Spearman's rank correlation coefficient between the therapy-induced change in IL-12/18-induced IFNγ-positive NK cells and the change in IL-12/18-induced CD69-positive NK cells was determined. Change was defined as the absolute difference between percentages at baseline and at 6 months.

VIRAL LOAD REDUCTION IMPROVES IFNγ PRODUCTION BY CD56^{dim} NK CELLS AND IS CORRELATED WITH INCREASED EX VIVO ACTIVATION. To examine whether these phenotypic changes were paralleled by functional alterations, PBMC obtained at baseline and at 6 months during therapy were simultaneously stimulated with IL-12 and IL-18 to investigate IFNγ production. Viral load reduction significantly enhanced the frequency of IFNγ producing NK cells (FIGURE 6A), which was mainly due to the 1.4-fold improved response of the CD56^{dim} NK cell subset (FIGURE 6B). Interestingly, the therapy-induced increase in IFNγ producing cells strongly correlated with the therapy-induced change in IL-12/IL-18-induced NK cell activation *ex vivo*, as indicated by the improved IL-12/IL-18-induced expression of CD69 (FIGURE 6C).

THE ABILITY OF NK CELLS TO PRODUCE CYTOKINES IS DEPENDENT ON THE CAPACITY TO BECOME ACTIVATED. The finding that the induction of CD69 expression upon *ex vivo* stimulation strongly correlated with the capacity to produce IFNγ led to the hypothesis that NK cells from chronic HBV patients have an impaired capacity to respond to activating signals. Indeed, in comparison to healthy controls, $CD56^{dim}$ but not $CD56^{bright}$ NK cells of patients with chronic HBV infection were less able to induce CD69 expression upon stimulation (FIGURE 7A). Within the patient NK cell population that expressed CD69, a similar frequency of IFNγ producing NK cells was found as in controls (FIGURE 7B). These data confirm that *de novo* NK cell activation is necessary for cytokine production[7, 27] and suggests that the capacity to become *de novo* activated is diminished in patients with chronic HBV infection.
Interestingly, viral load reduction increased the frequency of NK cells able to upregulate CD69 upon IL-12/18 stimulation (FIGURE 7C). Again, NK cells that gained CD69 expression, showed a similar frequency of intracellular IFNγ positive cells (FIGURE 7D). The enhanced capacity to get *de novo* activated was most pronounced for $CD56^{dim}$ NK cells and demonstrated a strong correlation with the altered capacity to produce IFNγ (FIGURE 7E). Comparison with clinical parameters revealed that not viral load, but serum ALT levels significantly correlated with *de novo* activation (FIGURE 7F) and function (data not shown).
These data indicate that anti-viral therapy in patients with chronic HBV infection enables more NK cells to become *de novo* activated leading to an improved capacity to produce IFNγ.

FIGURE 7. IMPAIRED *DE NOVO* ACTIVATION RESULTING IN DIMINISHED CYTOKINE PRODUCTION BY NK CELLS CORRELATES WITH HEPATIC DAMAGE/INFLAMMATION IN PATIENTS WITH CHRONIC HBV



A B: NK cells present in IL-12/18 stimulated fresh PBMC derived from 25 healthy controls (HC) and 40 patients with chronic HBV infection (HBV) (see TABLE 1) were analysed for the expression of CD69 and IFNγ. Data demonstrate the percentage of CD69 expressing cells within the two NK cell subsets (A) and the percentage of IFNγ producing cells within the CD69-positive NK cells for both subsets (B) and are presented in box-and-whisker plots. **P<.01*

C D: Cryopreserved PBMC derived from patients at baseline (0) and at 6 months of therapy (6) were thawed and stimulated with IL-12/18 in parallel. NK cell subsets were analysed for the expression of CD69 and IFNγ. Data are demonstrated as described in AB.. **P<.05*

E F: Spearman's rank correlation coefficients between the therapy-induced changes in IL-12/18-induced CD69-positivity and IFNγ expression in CD56dim NK cells (E) and the therapy-induced changes in IL-12/18-induced CD69-positivity and hepatic inflammation/damage reflected as changes in serum ALT values (F), were determined.

DISCUSSION

Natural killer (NK) cells play a major role in anti-viral immunity as first line defense and regulation of virus-specific T cell responses. Originally, CD56^{bright} NK cells were regarded as the prime cytokine producing subset, whereas CD56^{dim} NK cells were held responsible for cytotoxicity[12]. Since hepatocytes, the major target of HBV, are known to be resistant for perforin/granzyme-mediated killing[25], the production of anti-viral cytokines is of major importance for combating viruses that predominantly infect hepatocytes[2, 19].

In line with previous studies[3, 27] but in contrast to others[30], we show that total NK cell percentages remain similar to controls. The current study shows that the cytotoxic capacity of NK cells is retained in patients with chronic HBV infection. We confirm that CD56^{bright} NK cells are relatively better at cytokine production (even up to 90% is positive for IFNγ) than their CD56^{dim} counterparts. However, upon stimulation with IL-12/IL-18 as well as PMA/Ionomycin, the majority of IFNγ producing NK cells is CD56^{dim}. We demonstrated that NK cell activation and IFNγ production, especially within the CD56^{dim} NK cell population, are strongly hampered in chronic HBV. Although anti-viral therapy enhanced the activation state of CD56^{bright} NK cells *in vivo*, it mainly affected the function of CD56^{dim} NK cells leading to an increased capacity to produce IFNγ as a result of an increased ability of CD56^{dim} NK cells to become activated *de novo*.

We demonstrated that independent of the stimulus used, NK cells derived from chronic HBV patients did not become activated to the same extent as NK cells derived from healthy controls. Once NK cells became activated as defined by CD69 positivity, they produced IFNγ equally well. Therefore, it seems that NK cells from chronic HBV patients have an impaired capacity to become activated *de novo*. Anti-viral treatment not only reduced viral load, but also lowered serum ALT levels i.e. hepatic inflammation. The therapy-induced change in serum ALT levels correlated with the improved ability of CD56^{dim} NK cells to get activated *de novo* which on its turn correlated with the improved production of IFNγ. It is maybe not surprising that viral load reduction affected IFNγ production by CD56^{bright} NK cells to a lesser extend since

most of these cells already produced IFNγ upon stimulation. The finding that viral load reduction enables NK cells to respond better to immune stimulating agents (e.g. IL-12, IL-18) may be helpful in the design of novel therapeutic strategies. Like IL-12 and IL-18, IFNα is a cytokine known to activate NK cells[4, 31]. Whether the improved anti-viral function of NK cells upon viral load reduction could also increase rates of definite viral clearance when combined with the immunomodulatory properties of exogenous IFNα requires further investigation.

The normal cytotoxic function with the defective production of cytokines by NK cells in unfractionated PBMC was recently observed by others[39]. By performing cytokine production assays with purified NK cells, we now really show that this impaired cytokine production is at least due to a defect in the NK cell pool. Interestingly, the more pronounced defect in NK cell function observed in total PBMC cultures compared to purified NK cell cultures may point towards a role for accessory cells. It has been shown that in PBMC cultures, specifically impaired dendritic cells could be responsible for the defective cytokine production by NK cells[22, 49]. This is of importance since also in chronic HBV, dendritic cells (DC) are known to be functionally impaired[39]. Moreover, the NK receptor NKp30 plays an important role in DC-NK crosstalk[16] and was significantly downregulated in chronic HBV patients, which was surprising since expression of NKp30 was not found different in earlier smaller studies[39]. Although the frequency of NKp30 expressing cells correlated with viral load in patients, this receptor does not seem directly involved in the improved cytokine production upon treatment since therapy-induced viral load reduction did not alter NKp30 expression. The exact role of NKp30 and DC-NK cell crosstalk in chronic HBV remains to be further elucidated.

Another remarkable finding is the decreased CD69, but increased HLA-DR expression, again especially on the $CD56^{dim}$ NK cells in chronic HBV patients. This altered activation status together with the altered expression of stimulatory and inhibitory receptors, such as NKp30 and NKG2A, may reflect (split) exhaustion as has been described for T cells during chronic viral infection[1, 34, 35]. However, molecular mechanisms underlying (split) exhaustion are still not clear and not documented for NK cells. Although we could not establish significant correlations

between the expression of NK cell receptors, such as NKG2A and NKp30, and activation status or function, it does not exclude a role for these receptors or their ligands in the regulation of NK cell activation and/or function. Since NKG2A triggering has been shown to inhibit IFNγ production[31], it is tempting to speculate that in chronic HBV infection, increased expression of NKG2A prohibits NK cells from being activated and subsequent function. In that respect, the downregulation of NKG2A upon viral load reduction as demonstrated here, may be in part responsible for the improved NK cell function.

Next to NKp30 and NKG2A also CD16, albeit to a limited extent, was significantly differently expressed in chronic HBV. However, to our knowledge CD16 expression has not been linked to IFNγ and/or TNFα production. CD16 is often used to discriminate between the $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets, since CD16 is almost exclusively expressed on $CD56^{dim}$ NK cells[11]. Although we did not observe a skewing in NK cell subsets in chronic HBV, as also previously reported by others[29], it is interesting to note that chronic HIV infection is associated with the decline of $CD56^{dim}$CD16+ and increased terminal differentiation of various lymphocyte subsets, thereby supporting the hypothesis of exhaustion[21]. More recently, CD94 was also described as a helpful marker to identify a differentiation stage between the different NK cell subsets[18]. Also according to differentiation stages/NK cell subsets described by Yu et al[41] we did not observe significant differences between chronic HBV patients and healthy controls (data not shown).

Although we cannot draw firm conclusions due to our experimental setup, it seems that viral load reduction did not completely restore cytokine production by NK cells to healthy control levels. In HIV exposed individuals, upregulation of CD69 was found in those who remained uninfected supporting the notion that, among other factors, an increase of NK cell activation is associated with definite clearance[33]. Furthermore, sustained viral response in chronic HCV infection is augmented by the increase of $CD56^{dim}$CD69+ NK cells[25]. The finding that anti-viral therapy of the chronic HBV patients investigated here did neither alter the expression of CD69 on $CD56^{dim}$ NK cells, nor changed the expression levels of other activation markers such as HLA-DR, CD11a and CD11c on

NK cells, may indicate that a certain level of dysfunction/exhaustion still exists, which prohibits the development of effective HBV-specific immunity. In conclusion, although the NK cell cytotoxic capacity is retained, the defect in the activation of NK cells and the subsequent reduced capacity to produce anti-viral and Th1-skewing cytokines may play a role in HBV persistence. Restoration of this cytokine producing capacity by NK cells as achieved by viral load reduction could therefore be of importance for the definite clearance of the virus.

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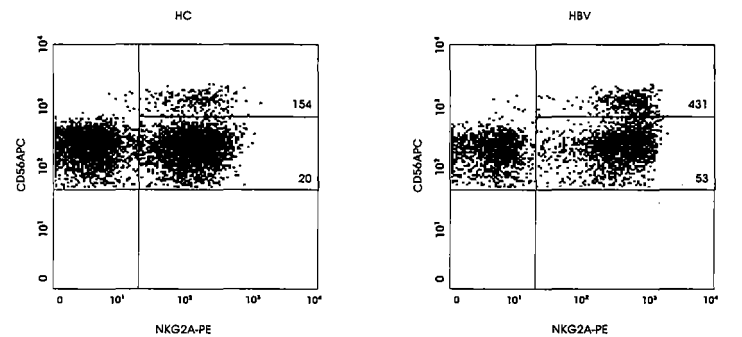
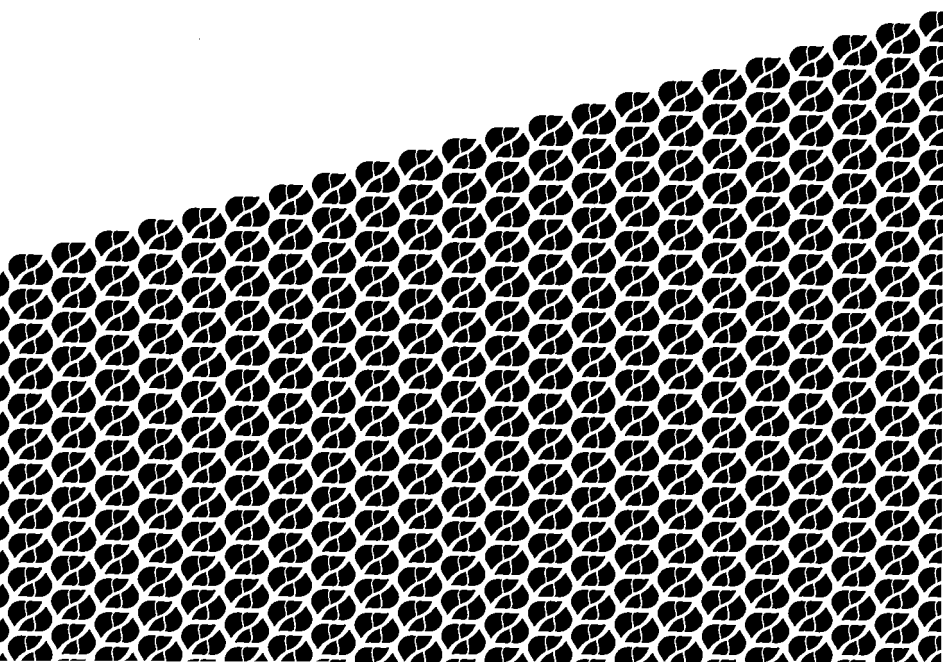
SUPPL. FIGURE 1





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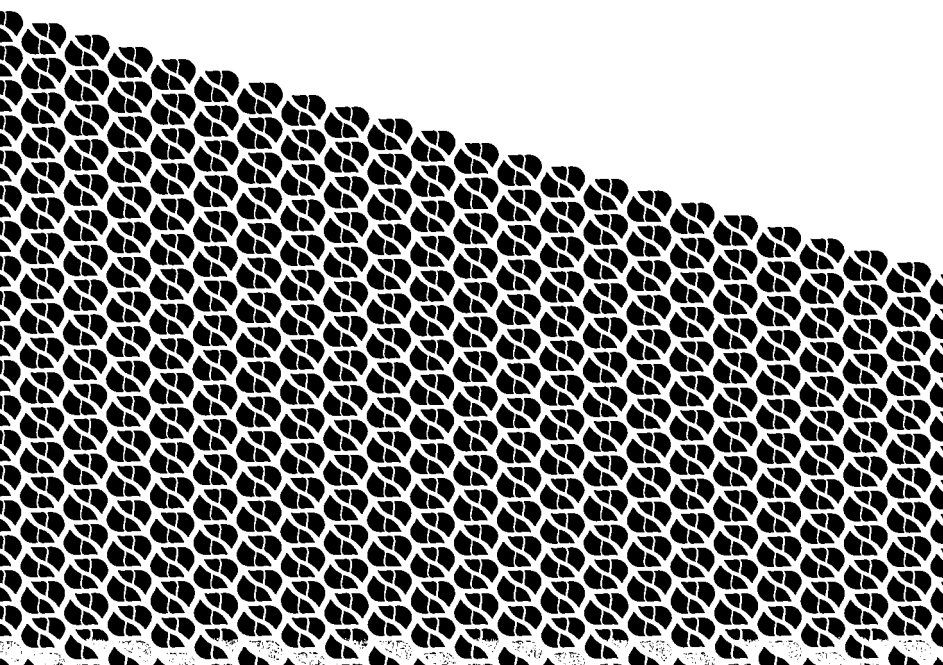


CHAPTER 3

HEPATITIS B VIRUS SUPPRESSES THE FUNCTIONAL INTERACTION BETWEEN NATURAL KILLER CELLS AND PLASMACYTOID DENDRITIC CELLS

ABSTRACT

BACKGROUND Natural killer cells (NK) are one of the key players in the eradication and control of viral infections. Infections with the hepatitis B virus (HBV) may lead to persistence in a subgroup of patients, and impaired NK cell functions have been observed in these patients. Crosstalk with other immune cells has been shown to modulate the function of NK cells.

OBJECTIVE This study aimed to investigate the functional crosstalk between NK cells and plasmacytoid DC (pDC) and its modulation by HBV.

METHODS Healthy human peripheral blood-derived NK cells and pDC were purified and co-cultured in the presence or absence of HepG2.2.15-derived HBV under various *in vitro* conditions. The functionality of NK cells was assessed by evaluation of activation markers, cytokine production, and cytotoxicity of CFSE-labelled K562 target cells by flow cytometry or immunoassays. Additionally, the crosstalk was examined using NK and pDC from chronic HBV patients.

RESULTS The activation of NK cells in co-cultures with pDC, as demonstrated by CD69, CD25 and HLA-DR, was not affected by the presence of HBV. Similarly, when co-cultured with pDC, the cytotoxic potential of NK cells was not influenced by HBV. However, HBV significantly inhibited pDC-induced IFN-γ production by NK cells both in the presence and absence of CpG. Since HBV did not affect cytokine-induced IFN-γ production by NK cells cultured alone, the suppressive effect of HBV on NK cell function was mediated via interference with pDC-NK cell interaction.

CONCLUSION In contrast to other viruses, HBV does not activate pDC-NK cell interaction but inhibits pDC-induced NK cell function. In parallel with NK cells of chronic HBV patients, which show diminished cytokine production with normal cytotoxicity, HBV specifically suppressed pDC-induced IFN-γ production by NK cells without affecting their cytolytic ability. These data demonstrate that HBV modulates pDC-NK cell crosstalk, which may contribute to HBV persistence.

INTRODUCTION

More than 400 million people are chronically infected with hepatitis B virus (HBV) and are at risk of developing liver cirrhosis or hepatocellular carcinoma[1]. Chronic HBV infection is characterized by inadequate innate immune response towards the virus with respect to cytokine production and T cell activation[2-4]. Innate immunity plays a crucial role, not only in controlling virus infection in the primary stage, but also in instructing the adaptive immune system to initiate a specific response against the virus[5].

Natural killer (NK) cells are important innate immune cells in anti-viral immunity as they are able to kill virus-infected cells and produce key inflammatory cytokines[6-7]. NK cell-derived cytokines, notably IFN-γ, are important for promoting Th1 cell development, and subsequent cytotoxic $CD8^+$ T cell response[8] as well as inhibiting viral replication. NK cell activation and function are influenced by the expression of inhibitory and activating receptors on their cell surface[9], the cytokine milieu, and the interaction with other immune cells, including dendritic cells (DC)[10]. Bidirectional crosstalk between NK cells and DC has been shown to be crucial for the regulation of both innate and adaptive immunity against infections[11-13]. Their interaction, mediated via cytokines or cell-cell contact, results in activation and enhanced function of both cell types, and can be facilitated by innate immune receptors, such as Toll-like receptors (TLR). Plasmacytoid DC (pDC) are a specialized DC subpopulation that produces high levels of type I interferons, and subsequently play an important role in antiviral immunity by direct inhibition of viral replication[14]. In addition, pDC also activate other lymphocytes, including NK cells, allowing further priming and regulation of anti-viral immunity[15]. Regarding the interplay between pDC and NK cells, it was recently shown that TLR-stimulated pDC recruit and activate NK cells to become more cytotoxic *in vivo*[16].

In recent years, a number of groups have reported on the functionality of NK cells and pDC in chronic HBV patients. NK cells from chronic HBV patients display a normal cytolytic activity, but a markedly diminished ability to produce IFN-γ[17-19]. Regarding pDC function, we and others reported that chronic HBV patients display a diminished capacity to

produce IFN-α (3, 20-23). The mechanisms underlying dysfunction of pDC and NK cells in chronic HBV infection are not fully understood, and no information is available on the modulation by HBV on the interplay between NK cells and pDC.
In this study, the effect of HBV on the functional interaction between NK cells and pDC was investigated. We observed that HBV did not enhance NK cell activation in cultures containing pDC and NK cells. However, HBV suppressed their functional crosstalk, leading to diminished pDC-induced IFN-γ secretion by NK cells without affecting NK cell cytotoxicity. This defective NK cell function due to crosstalk with pDC may represent an important immune escape strategy for the virus, explaining the enhanced risk for persistent HBV infection.

MATERIALS AND METHODS

CELL ISOLATION FROM PERIPHERAL BLOOD. Peripheral blood-derived NK cells and pDC were purified from buffy coats of 15 healthy donors (Sanquin, the Netherlands) or from heparin blood collected from 6 chronic HBV patients. Peripheral blood mononuclear cells (PBMC) were isolated by ficoll separation (Ficoll-Paque™ plus, Amersham Biosciences, UK). NK cells were negatively isolated by magnetic cell sorting according to manufacturer's instructions (Miltenyi Biotec, Germany). Autologous pDC were isolated from the same buffy coat using positive magnetic selection with BDCA-4 antibody (AD5-17F6) (Miltenyi Biotec), followed by cell sorting (FACS Aria; BD Biosciences, Sunnyvale, CA) to get more than 98% pure pDC. All experiments were conducted in triplicate. The data represented are the mean of these triplicates x number of experiments as stated in the legends.
Chronic HBV infected patients (n=6) had HBV DNA detectable by PCR and were positive for serum HBV surface antigen for at least 6 months. None of the patients were co-infected with other viruses, or were previously treated for HBV infection. The institutional ethical review board

of the Erasmus MC approved the clinical protocols, and written informed consent was obtained from all individuals prior to their inclusion.

CELL CULTURE. Freshly isolated NK cells and pDC were co-cultured in RPMI 1640 containing 10% FCS in 96-well round-bottom plate for 48h, at a ratio of NK/pDC= 5:1 (NK cell: 10^5 cells/well; pDC: $2x10^4$ cells/well). Cultures were set up in the presence or absence of the following stimuli: CpG-2336 (class A; 10 μg/ml, Coley Pharma, Düsseldorf, Germany), and HBV (40 geq/cell). HepG2.215-derived HBV particles were generated and purified as described before[24]. Culture medium was supplemented with 20 ng/ml IL-3 (Miltenyi Biotec) for NK-pDC co-cultures and control conditions. In addition, as positive controls, NK cells were treated with IL-2 (Strathman, Germany) or with IL-18 (100 ng/ml; MBL International Corporation, Japan) and IL-12 (10 ng/ml; Miltenyi Biotec) or IFN-α2b ($1.25x10^4$ IU/ml; Intron A, Schering Plough, USA).

PHENOTYPE AND INTRACELLULAR MOLECULES ANALYSIS. To measure the expression of activation-related surface molecules on NK cells and pDC, cells were co-cultured for 48h and evaluated for expression of CD69, CD25 and HLA-DR expression on NK cells using anti-CD69 (L78, BD), anti-CD25 (2A3), anti-HLA-DR (LN3, eBioscience), and anti-CD56 (MY31, BD). NK cells were positively gated on the basis of CD56 expression. For the analysis of pDC activation, CD83 and CD86 expression was evaluated using anti-CD83 (HB15e, Caltag), anti-CD86 (IT2.2, Biolegend), and anti-CD123 (AC145, Miltenyi Biotec) to identify pDC. Corresponding isotype antibodies were used to determine background staining. All samples were acquired by flowcytometry (FACS Canto II) and analysed by FACS Diva software (both from BD).

CYTOKINE PRODUCTION ASSAY. Supernatants were collected after co-culture for 48h. IFN-α and IFN-γ levels in supernatant were determined by ELISA according to the manufacturer's instructions (IFN-α, eBioscience; IFN-γ, Bender MedSystems GmbH, USA). Besides ELISA, also intracellular cytokine staining using flow cytometry was used to assess IFN-γ production in NK-pDC co-cultures, as was previously described[17].

CELL-MEDIATED CYTOTOXICITY ASSAY. Following culture for 48h, NK cells were harvested and incubated with $5x10^3$ CFSE labeled K562 cells (10 nM; Molecular Probes, USA) at different effector/target ratios (1/1.25, 1/2.5, 1/5, 1/10). After incubation for 4h, cells were harvested, washed, stained with 7-aminoactinomycin D (7-AAD, eBioscience) and analyzed by flow cytometry. The percentage of killing of K562 cells in the assay was calculated by determining the percentage of $7\text{-AAD}^+\text{CFSE}^+$ double positive cells. Background killing, i.e. the percentage of $7\text{-AAD}^+\text{CFSE}^+$ K562 cells in the absence of NK cells was subtracted.

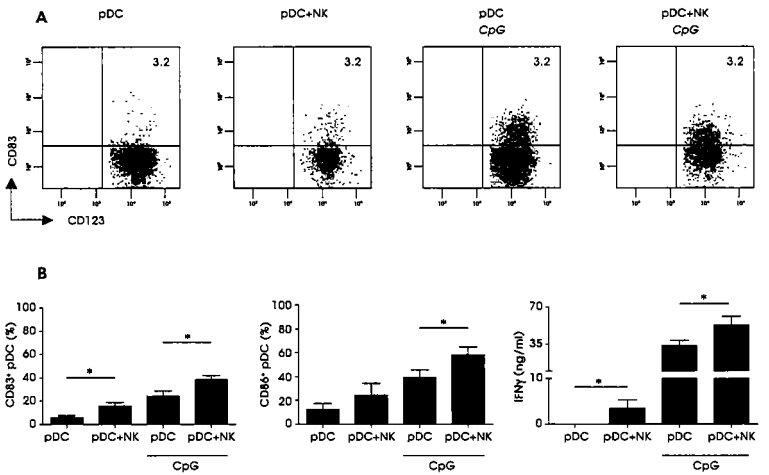
STATISTICAL ANALYSIS. Data are expressed as mean ± standard error of the mean (SEM) and analysed with Prim 5.0 (GraphPad software, USA). Statistical significance ($p<.05$) was performed between paired samples using the Wilcoxon signed rank test.

RESULTS

THE PRESENCE OF NK CELLS IN CO-CULTURE WITH pDC PROMOTES pDC ACTIVATION. We previously demonstrated that HBV inhibits pDC function in *in vitro* assays[29]. To examine the consequences of the impaired pDC activation by HBV, we evaluated the functional interaction between pDC and NK cells. We first examined –in the absence of HBV- whether the presence of NK cells affects the activation status of pDC by *in vitro* co-culture of freshly isolated healthy peripheral blood NK cells and autologous pDC in the presence or absence of the TLR9 ligand CpG. As shown in FIGURE 1A and 1B, the consequence of co-culture of pDC and NK cells in the absence of additional stimuli was a more activated phenotype of pDC as demonstrated by enhanced expression of CD83 and CD86. Furthermore, co-culture of NK cells with CpG-stimulated pDC, also resulted in higher levels of expression on pDC of CD83 and CD86 as compared to cultures without NK cells. The capacity to produce large amounts of IFN-α is a hallmark of pDC

function and important for the regulation of anti-viral immunity, including NK cell function. In line with the augmented expression of activation markers and in accordance with previous findings[15], the presence of NK cells in cultures containing pDC significantly enhanced the ability of pDC to produce IFN-α, either spontaneous or upon additional triggering of TLR9 (FIGURE 1B).

FIGURE 1. THE PRESENCE OF NK CELLS IN CO-CULTURE WITH pDC PROMOTES pDC ACTIVATION

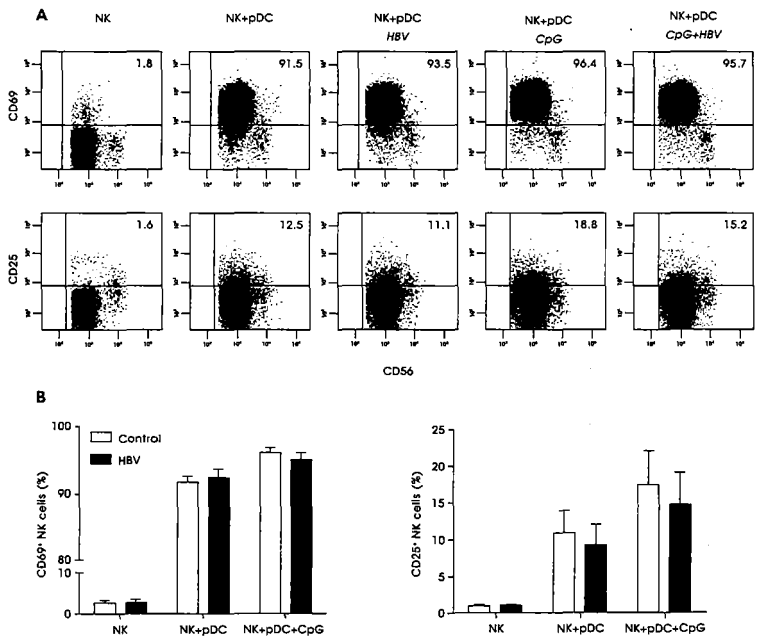


pDC were cultured alone or co-cultured with NK cells either in the presence or absence of CpG for 48h. After co-culture, the percentage of pDC expressing CD83 and CD86 were assessed by flow cytometry. One representative experiment (A) and the mean±SEM of the IFN-α levels determined by specific ELISA in supernatant of 15 independent experiments are shown (B). * P<0.05

HBV HAS NO EFFECT ON pDC-INDUCED NK CELL ACTIVATION AND CYTOTOXICITY. Accompanied by enhanced activation of pDC in co-cultures with NK cells, we also examined the activation state of NK cells in these cultures by evaluating the expression of CD69 and CD25. Both activations were highly upregulated in co-culture with pDC in the

absence of additional stimuli. This upregulation was more pronounced for CD69 than CD25, since about 90% of NK cells, especially those within the CD56^{dim} NK cell subpopulation, expressed CD69 following co-culture with pDC (FIGURE 2A). Addition of CpG, to activate pDC, only resulted in a weak upregulation of CD69 expression. This small effect of CpG was via pDC since no changes in CD69 expression on NK cells cultured alone were observed (data not shown). Next, we examined the ability of HBV to modulate the pDC-NK cell interaction. As shown in FIGURE 2A and 2B, irrespective of the presence or absence of additional pDC activation

FIGURE 2. HBV HAS NO EFFECT ON pDC-INDUCED NK CELL ACTIVATION

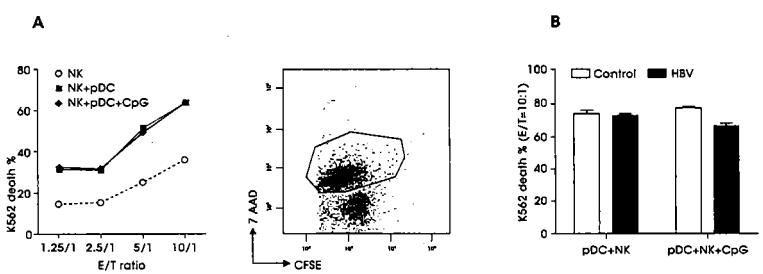


NK cells were cultured alone or co-cultured with pDC in the presence or absence of CpG, and in the presence or absence of HepG2.2.15-derived HBV. After 48h culture, CD69 and CD25 expression on NK cells were assessed by flow cytometry. Dotplots are shown of a representative experiment (A) and the mean±SEM of five independent experiments with different donors (B).

by CpG, addition of HBV to the co-cultures did not affect the expression of CD69 expression on NK cells. Similar findings were observed for the effect of HBV on the expression levels of CD25 and HLA-DR on NK cells (FIGURE 2B, and data not shown). Besides the expression of activation markers, also the effect of HBV was examined on NK cell cytotoxicity in co-cultures with pDC. As demonstrated in FIGURE 3A, the relatively low cytotoxicity of K562 target cells by NK cells alone was significantly increased by co-culture with pDC, irrespective of the presence of CpG (FIGURE 3A). However, the presence of HBV in pDC-NK cell co-cultures did not modulate the pDC-induced killing activity of NK cells. The assays were conducted at increasing E/T ratios, and no effect of HBV on K562 cytotoxicity was observed at any of these conditions (FIGURE 3B, and data not shown).

These data therefore show that despite our previous findings that HBV inhibits the function of pDC[29], no effect of HBV on pDC-induced NK cell activation or cytotoxicity was observed in *in vitro* co-culture experiments.

FIGURE 3. CYTOLYTIC ABILITY OF NK CELLS IS NOT INFLUENCED BY HBV



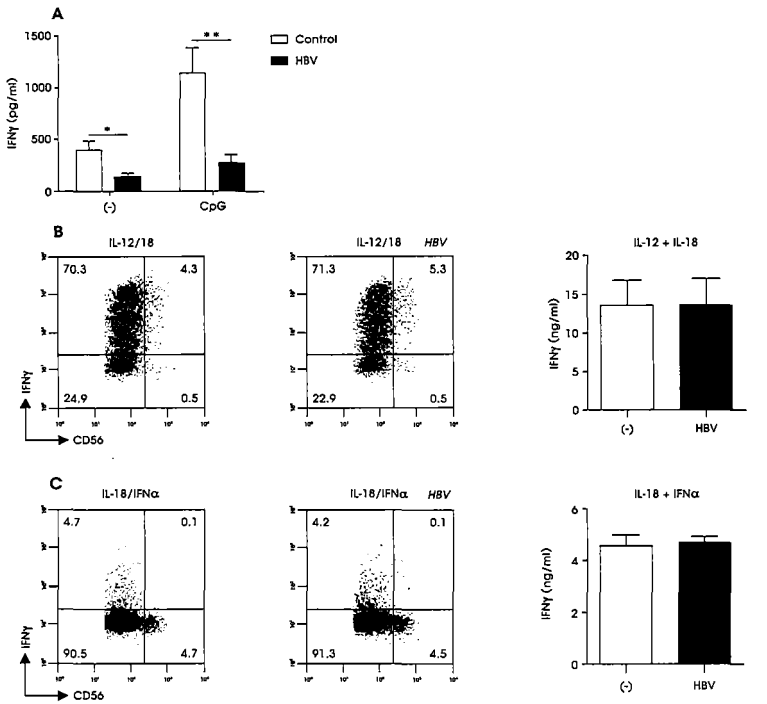
NK cells were cultured alone or co-cultured with pDC in the presence or absence of CpG, and in the presence or absence of HepG2.2.15-derived HBV. After 48h culture, cells were harvested and incubated with CFSE-labeled K562 cells at the indicated effector-target (E:T) ratios. After 4h, K562 cell death was determined by analyzing 7-AAD positive cells within the population of $CFSE^+$ cells. A representative experiment (A) and the mean±SEM K562 cell death at an E:T ratio of 1:10 of three independent experiments with different donors (B) are shown.

HBV INHIBITS pDC-INDUCED IFN-γ PRODUCTION BY NK CELLS. Besides their ability to induce cytotoxicity and kill certain target cells, NK cells can also produce high levels of IFN-γ upon viral infection, which may lead to priming or activation of innate cells, and modulate adaptive immune responses[25]. As expected, non-stimulated NK cells did not produce detectable amounts of IFN-γ, however, in the presence of pDC, relatively low levels of IFN-γ by NK cells were observed, which were further enhanced upon CpG stimulation of the pDC population (from 378 to 1135 pg/ml IFN-γ; FIGURE 4A). Next, we examined the effect of HBV addition to the co-cultures of pDC and NK cells, and observed that the addition of HBV resulted in a significant reduction of IFN-γ production by NK cells when these cells were co-cultured with resting as well as with CpG-stimulated pDC (FIGURE 4A). To investigate whether this specific impairment in NK cell-derived IFN-γ production was due to an effect of HBV on pDC-NK cell crosstalk or due to the direct interference of HBV on NK cells, IFN-γ was induced in purified NK cells by either a cocktail of cytokines consisting of IL-12 and IL-18, or of IL-18 and IFN-α. Comparison of cytokine-stimulated NK cells activated either in the presence or absence of HBV demonstrated that both the frequency of IFN-γ-producing NK cells, and the levels of IFN-γ produced by NK cells were similar (FIGURE 4B and 4C). These data show that HBV does not directly affect IFN-γ production by cytokine activated NK cells, but exerts its inhibitory effect via interference with pDC-NK cell crosstalk.

HBV DOES NOT MODULATE NK CELL NUMBERS IN VITRO CO-CULTURE. To further examine how HBV exposure results in inhibition of pDC-induced IFN-γ production by NK cells, we determined the absolute cell numbers of NK cell in co-culture experiments. As shown in FIGURE 5, the presence of pDC enhanced the absolute number of NK cells, which was reported previously[26]. Addition of HBV did not affect the absolute number NK cells after culture for 48h, suggesting that pDC-induced IFN-γ production by NK cells are not likely due to a strong effect on cell viability or proliferation.

pDC-INDUCED IFN-γ PRODUCTION BY NK CELLS IS IMPAIRED IN CHRONIC HBV PATIENTS. Since HBV impairs pDC-induced NK cell derived IFN-γ levels *in vitro*, we wondered whether this was also reflected

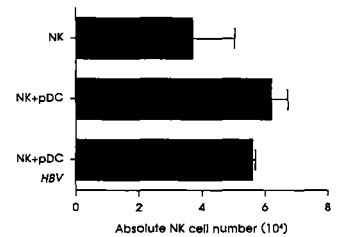
FIGURE 4. HBV SUPPRESSES pDC-INDUCED IFN-γ PRODUCTION BY NK CELLS



NK cells were co-cultured with pDC with or without CpG, in the presence or absence of HepG2.2.15-derived HBV. The IFN-γ levels were measured in supernatant by ELISA are presented as mean±SEM of fifteen independent of experiments (A). * P<0.05, ** P<0.01. Moreover, purified NK cells, cultured alone, were stimulated with IL-12 combined with IL-18 (B), or IL-18 and IFN-α (C) in the presence or absence of HBV. Representative dotplots showing intracellular cytokine staining by flow cytometry, and IFN-γ levels as assessed by ELISA (shown as mean±SEM) of 8 donors are expressed. * P< 0.05, ** P< 0.01.

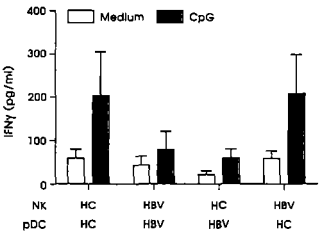
by the functionality of these cells when obtained from chronic HBV patients. As shown in FIGURE 6, no differences in spontaneous production of IFN-γ were observed when NK cells and pDC purified from healthy individuals were compared to NK cells and pDC purified from chronic HBV patients in co-cultures. However, when upon stimulation with CpG, the levels of IFN-γ detected in the NK-pDC co-culture were reduced when NK

FIGURE 5. HBV DOES NOT AFFECT NK CELL NUMBERS IN CO-CULTURE EXPERIMENTS



NK cells and pDC were co-cultured as previously described. After cell culture, total number of living cells in each well was determined by counting trypan blue (TB) cells under the microscope. The percentage of CD56⁺ CD3⁻ cells were detected by flow cytometry. and the absolute number of NK cells was thus established.

FIGURE 6. pDC-INDUCED IFN-γ PRODUCTION BY NK CELLS IS IMPAIRED IN CHRONIC HBV PATIENTS



NK cells from 6 patients were co-cultured with pDC from healthy donors, or vice versa. After co-culture in the presence or absence of CpG, IFN-γ production in supernatant was easured by specific ELISA. Data are shown as mean±SEM of 6 independent experiments with different subjects.

cells and pDC were both obtained from patients as compared to healthy individuals. Next, NK cells from healthy individuals were co-cultured with CpG-activated pDC from chronic HBV patients. In this culture system, the IFN-γ levels were reduced as compared to the homologous healthy control co-cultures and similarly low as the chronic HBV co-cultures. In contrast, when NK cells from a chronic HBV patient were cultured CpG-activated pDC from a healthy individual, the IFN-γ production was similarly high as the homologous co-culture system where both pDC and NK cells originated from healthy individuals. Our findings indicate that ability of NK cells obtained from chronic HBV patients to produce IFN-γ is reduced as an indirect consequence of the impaired function of pDC.

DISCUSSION

Here we studied the effect of HBV on NK cells during the crosstalk with pDC. We showed that the ability of NK cells, upon interaction with pDC, to produce effector cytokines is suppressed upon exposure to HBV, but not their activation or cytotoxicity. Furthermore, our findings indicate that HBV did not directly inhibit NK cell activity, but indirectly via its effect on pDC. These data could not only partially explain the impaired innate immune response upon HBV infection, but may also provide a mechanism for the observed functional dichotomy of NK cells circulating in patients with chronic HBV.

In line with previous reports, we showed that crosstalk between pDC and NK cells results in activation of both cell types, as we showed by upregulation of activation markers and cytokine production. In a highly controlled *in vitro* cell culture system, the effect of HBV on a mixed culture of stimulated pDC and NK cells was a reduced IFN-γ production by NK cells, but unaffected cytotoxicity and activation of NK cells when exposed to HBV. No data support in vitro infection of pDC or NK cells[4]. These findings obtained from *in vitro* cultures are reminiscent of previous reports by our group and others that showed that NK cells from chronic HBV patients display functional dichotomy, with retained cytotoxic capacity and strongly hampered IFN-γ production[17-18], which may have consequences for an effective antiviral immune response in chronic HBV patients.

In the present study, we also showed that NK cells themselves are not directly modulated by HBV, but indirectly as shown using sorted activated NK cells exposed to HBV. Furthermore, one of the cell types that may be responsible for the impairment of NK cell function upon expsoure to HBV is the pDC. In this, we found a more pronounced defect of NK cell derived-IFN-γ production using pDC from patients co-cultured with NK cells from healthy individuals, as compared to "healthy" pDC co-cultured with "patient" NK cells. This suggests that impairment of pDC function in patients leads a defect in NK cells as evidenced by reduced IFN-γ production. We previously showed that pDC exhibit phenotypical and functional impairment in patients with

chronic HBV infection[5]. Moreover, although HBV seems to be incapable to replicate in pDC, Xu et al. recently reported a decreased CpG-induced IFN-α production by pDC exposed to HBsAg[27]. This is in line with our recent published data, which showed that HBV interfered with TLR9-induced pDC function, resulting in dose-dependent inhibition of cytokine production and pDC maturation[29].

It has recently been found that pDC-dependent NK cell activity was impaired in viremic HIV patients[28, 29]. In contrast to our findings for HBV, the dysfunction in HIV patients was due to a non-responsiveness of NK cells to the cytokines IFN-α and TNF, as well as to a defect in secretion of IFN-α and TNF by pDC from HIV-infected individuals[28, 29]. Furthermore, *in vitro* exposure to exogenous HIV-1 Vpr protein has been found to interfere with pDC function as shown by suppressed type I IFN production, resulting in efficient inhibition of the pDC-NK cell interaction as shown by diminished IFN-α production[30]. It is important to mention that the impaired IFN-γ production by NK cells in the setting of chronic HBV infection is not an irreversible dysfunction. It was reported by Peppa et al., that lifting of the inhibition mediated by the immunosuppressive cytokines IL-10 or TGF-β restored the IFN-γ production by NK cells in chronic HBV patients[17]. In addition, we showed that therapy-induced viral load reduction in chronic HBV patients also restored the functionally impaired IFN-γ production by NK cells[17]. On the basis of our findings on the crosstalk between pDC and NK cells, it is tempting to investigate how modulation of pDC function in patients by, for instance, therapeutic intervention affects the NK cell compartment, and whether this leads to restoration of the impaired NK cells in chronic HBV patients.

In conclusion, our study demonstrated that HBV does not directly affect NK cells, but actively interferes with the functional interaction between NK cells and pDC, leading to reduced pDC-induced cytokine production by NK cells. The lack of immune activation combined with the interference with innate immunity may partially explain the development of HBV persistence.

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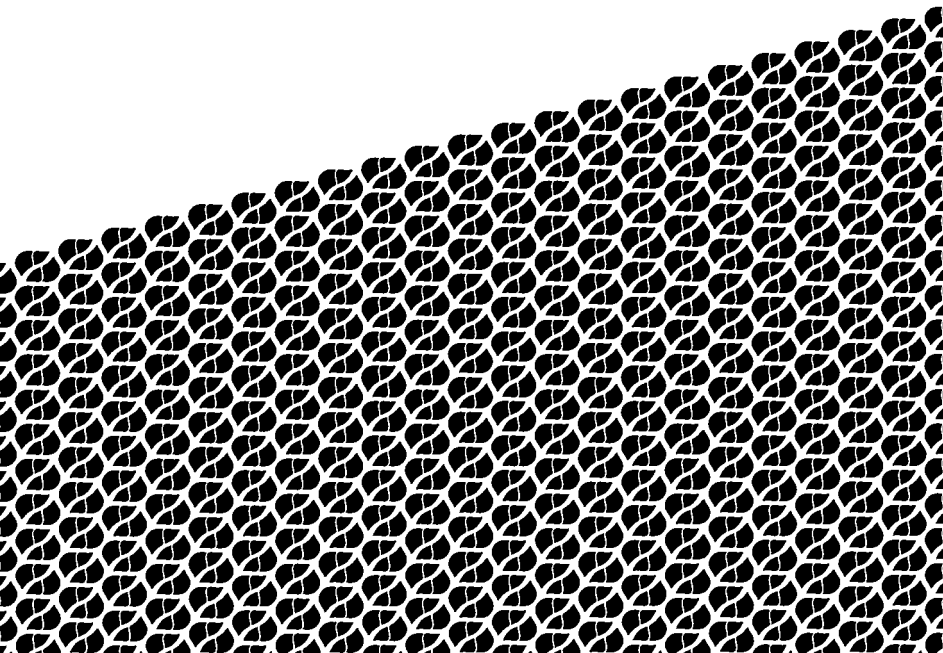

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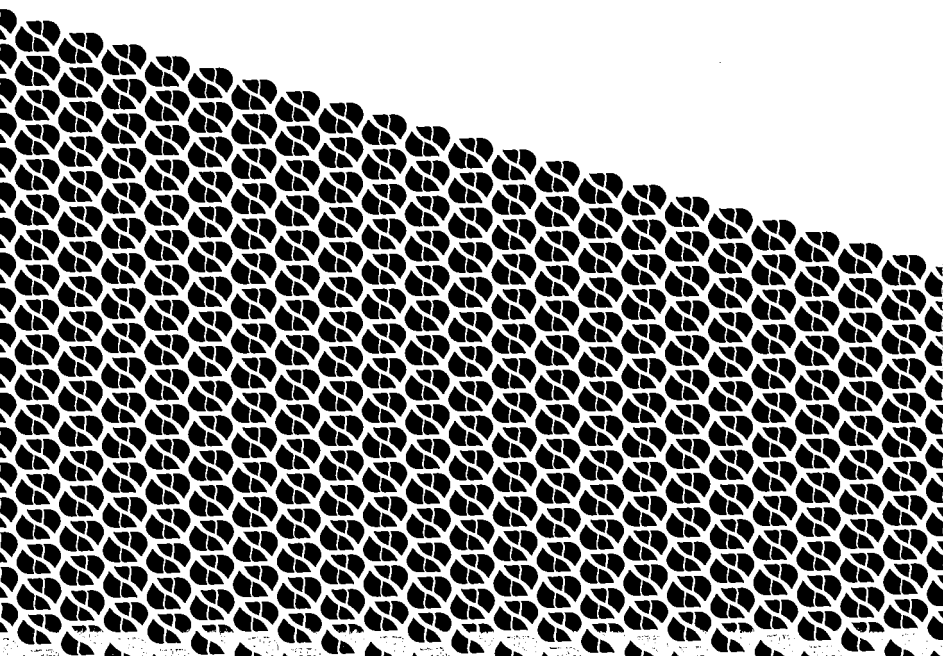


CHAPTER 4

RESTORATION OF TLR3-ACTIVATED MYELOID DENDRITIC CELL ACTIVITY LEADS TO IMPROVED NATURAL KILLER CELL FUNCTION IN CHRONIC HEPATITIS B

ABSTRACT

OBJECTIVE There is increasing evidence that the function of NK cells in patients with chronic hepatitis B (CHB) infection is impaired. The underlying mechanism for the impaired NK cell function is still unknown. Since myeloid dendritic cells (mDC) are potent inducers of NK cells, we investigated the functional interaction of mDC and NK cells in CHB and the influence of antiviral therapy.

DESIGN Blood BDCA1+ mDC and NK cells were isolated from 16 healthy controls, or 39 CHB patients at baseline and during 6 months of antiviral therapy. After activation of mDC with polyI:C and IFN-γ, mDC were co-cultured with NK cells. Phenotype and function were analysed in detail by flow cytometry and ELISA.

RESULTS Our findings demonstrate that on polyI:C/IFN-γ-stimulated mDC from CHB patients the expression of costimulatory molecules was enhanced, while cytokine production was reduced. In co-cultures of polyI:C/IFN-γ-stimulated mDC and NK cells obtained from CHB patients reduced mDC-induced NK cell activation (i.e. CD69 expression) and IFN-γ production was observed as compared to healthy individuals. Antiviral therapy normalized mDC activity since decreased expression of CD80 and CD86 on DC and HLA-E on NK cells was observed, while polyI:C/IFN-γ-induced cytokine production by mDC was enhanced. In parallel, successful antiviral therapy resulted in ameliorated mDC-induced NK cell activation and IFN-γ production.

CONCLUSIONS These data demonstrate that CHB patients display a diminished functional interaction between polyI:C/IFN-γ activated mDC and NK cells due to impaired mDC function, which can be partially restored by antiviral therapy. Enhancing this reciprocal interaction could reinforce the innate and thus adaptive T cell response, and may be an important step in achieving effective antiviral immunity.

INTRODUCTION

Chronic hepatitis B (CHB) is the result of an inadequate immune response against a persistent hepatotropic virus[39]. Research has traditionally been directed towards the ineffective HBV-specific T-cell response, while in recent years also innate immunity and its cellular components, like natural killer (NK) cells and dendritic cells (DC), received increasing attention[5]. It has been reported that both NK cells and DC are affected in patients with CHB. NK cells are important during chronic HBV infection by causing hepatocyte damage through apoptosis[12]. We and others recently reported that NK cell activation, as demonstrated by CD69 expression, and production of pro-inflammatory cytokines by NK cells such as IFN-γ are decreased in CHB patients, whereas direct cytotoxicity remains intact [25, 32]. This functional dichotomy may likely contribute to viral persistence. A role for IL-10 has been suggested in causing the NK cell impairment[26], although also negative regulation through enhanced expression of the inhibitory receptor NKG2A has been described[32].

NK cells are in close encounter with DC, and the outcome on NK cell function is dependent on reciprocal interaction with DC (9, 16, 28). There is evidence that the function of myeloid DC (mDC) in blood is impaired in patients with CHB[36]. Stimulated mDC secrete several cytokines, such as IL-6 and IL-12[14], that act as potent inducers of NK cell activation and proliferation. In turn, activated NK cells produce IFN-γ and TNF, which are directly involved in completing the maturation program of mDC[1]. NK cell activation is driven by a direct interaction between activated NK cells and mature mDC through attenuated expression of HLA-E, the ligand for NKG2A[19] and MHC-I molecules, the natural ligands for inhibitory NK receptors[22], and also through expression of activating receptor NKp30 on mDC[15]. In HIV infection, defective interaction through impaired function of NKp30 on mDC led to secretion of inadequate amounts of IFN-γ by NK cells[29] . Even though altered NKp30 expression is observed in acute liver failure[25, 37], little attention has been given to this interaction between mDC and NK cells in CHB. In this study we aimed to characterise the functional interaction between mDC and NK cells in CHB.

As liver inflammation and HBV-DNA load closely determine the immunoactive phase of CHB[39], the effect of the continuous presence of HBV-DNA on the immune system can be studied through antiviral therapy. Cytokine-producing capacities of both mDC and NK cells are partially restored upon antiviral therapy with nucleoside analogues[26, 34]. Of interest, we recently demonstrated that restoration of IFN-γ production was paralleled by enhanced activation of NK cells and a decrease of NKG2A expression[32]. We therefore hypothesize that antiviral therapy results in improved mDC-NK cell interaction through restoration of cytokine-mediated pathways of both mDC and NK cells, and through direct receptor-ligand expression. To study this, we examine the functional interaction of mDC and NK cells derived from a cohort of CHB patients treated with antiviral therapy.

PATIENTS AND METHODS

PATIENTS AND HEALTHY SUBJECTS. Peripheral heparinized blood samples were obtained from 39 patients with CHB for longitudinal analysis during antiviral therapy. Blood samples were obtained at baseline (t=0) and after 6 months (t=6) of antiviral therapy. Patients (HBeAg positive n=10, HBeAg negative n=29) were treated with nucleos(t)ide-analogues (NA) entecavir (0.5 mg o.i.d., n=22) or tenofovirdisoproxil (245 mg o.i.d., n=17) and all of them met the most recent EASL-guideline criteria for treatment of CHB. Patients receiving antiviral therapy within 6 months prior to treatment with NA were excluded. All patients were negative for antibodies against hepatitis C, hepatitis D and human immunodeficiency virus. An age and sex-matched control group comprised 16 healthy controls (HC). The study was approved by the local ethics committee, and all patients and healthy individuals gave informed consent before blood donation.

EXPRESSION OF INTRACELLULAR AND CELL SURFACE MOLECULES ON MDC AND ISOLATED NK CELLS BY FLOW CYTOMETRY. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Isopaque (GE Healthcare, Finland) gradient centrifugation. mDC were isolated from fresh PBMC by positive immunomagnetic selection using the mini-MACS-system (Miltenyi Biotec, Germany) according to the manufacturer's instructions. NK cells from PBMC were isolated by negative selection (purity and viability >95%) with an NK cell isolation kit (Miltenyi Biotec, Germany). For phenotypic analysis, incubation with a cocktail of fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin-chlorophyll-protein complex (PerCP) and allophycocyanin (APC)-conjugated monoclonal antibodies (mAbs) was performed. The mAbs directed against the following molecules were used: CD3 (UCHT1), CD69 (L78) and CD86 (2331) from BD Bioscience, Belgium; CD56 (N901), CD80 (mAB104), CD244 (C1.7) and CD48 (J4-57) from Beckman Coulter, USA; BDCA1 (AD5-8E7, Miltenyi Biotec, Germany); MHC class ABC (W6/32) and MIC-A (6D4) from Biolegend, USA; HLA-E (3D12, eBioscience, USA). As controls, cells were stained with corresponding isotype-matched control antibodies. Stained cells were analysed using a 4-color flow cytometer (FACS Calibur) and CellQuest software (both BD Bioscience, Belgium). Mean fluorescence intensity (MFI) and/or percentage positive cells were determined.

CO-CULTURE OF MDC AND NK CELLS. Freshly isolated mDC from CHB patients and HC were cultured in a concentration of $1x10^5$ in 200 µL culture medium (RPMI-1640, BioWhittaker/Cambrex, Belgium; 100 U/ml Penicillin and 100 µg/ml Streptomycin, from Invitrogen, USA; 10% Fetal Bovine Serum, Thermo Fisher Scientific, The Netherlands; 2 mM L-glutamine and 1 M Hepes from BioWhittaker/Cambrex, Belgium) with 50 ng/ml GM-CSF (Berlex, Germany); 20 µg/ml polyI:C (Sigma-Aldrich, Germany), and 250 U/ml IFN-γ (Strathman, Germany) in a round bottom 96-wells plate. Isolated mDC were also mono-cultured with 50 ng/ml GM-CSF as a positive control. After 18 hours, a small proportion of mDC was analysed by flow cytometry, the remainder of cells were washed. Fresh autologous NK cells, isolated synchronous (t=0 weeks or t=6 months) or thawed NK cells isolated 2 weeks prior to baseline, were added in a 1:5 mDC:NK cell ratio (>$20x10^3$ mDC/

well) in a round bottom 96-wells plate containing 200 µL per well. Isolated NK cells were also mono-cultured with 800 U/ml IL-2 (Strathman, Germany) as a positive control. After co-culture for 48 hours, cells were washed and processed for analysis. At all timepoints, supernatants were collected.

CYTOKINE PRODUCTION. Cytokine production by isolated mDC or NK cells from CHB patients and HC was determined after maturation for 18 hours with polyI:C and IFN-γ and after co-culture with isolated NK cells. Levels of IL-6, IL-12p40 and IL-12p70 and IFN-γ present in culture supernatants were determined by standard enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (eBioscience). Levels of IL-18 in culture supernatants were determined by flow-cytometry using labelled beads (human IL-18 Flowcytomix simplex, Bender MedSystems, USA).

STATISTICAL ANALYSIS. Data are expressed as mean±SEM or presented in bars, unless indicated otherwise. Data were analysed with Prism 5.0 (GraphPad software, USA) using the Mann-Whitney U test to compare variables between 2 independent groups, the Wilcoxon matched pairs test between paired variables and the Spearman's rank correlation coefficient test for non-parametric correlations. In all analyses, a two-tailed P-value of less than 0.05 was considered statistically significant.

RESULTS

DIMINISHED mDC-INDUCED ACTIVATION AND FUNCTION OF NK CELLS IN CHB. We recently showed that CD69 expression and IFN-γ production by NK cells is impaired in CHB patients[32]. Since the interplay between NK cells and mDC is crucial for their function, we decided to investigate the reciprocal functional interaction between mDC and NK cells of CHB patients and HC. For this purpose, we performed co-culture assays as described by Gerosa et al.,

for monocyte-derived DC and adjusted for ex vivo experiments as described in the Material and Methods section[16]. Group characteristics are shown in TABLE 1. For mDC-NK cell co-cultures, we activated the isolated mDC with polyI:C and IFN-γ prior to co-culture to prevent NK cell mediated killing of immature mDC[16]. As a result of the mDC-NK cell interaction, NK cells became activated as evidenced by upregulation of expression of the early activation marker CD69 on NK cells. After co-culture, NK cell viability was >90% whereas mDC viability was around 60%. mDC of CHB patients showed a 5.6 fold lower CD69 upregulation than HC (FIGURE 1A). This corroborates our previous findings that in CHB, baseline NK cells in unfractionated PBMC are less activated[32]. Besides lower activation levels, also NK cell derived IFN-γ levels detected in mDC-

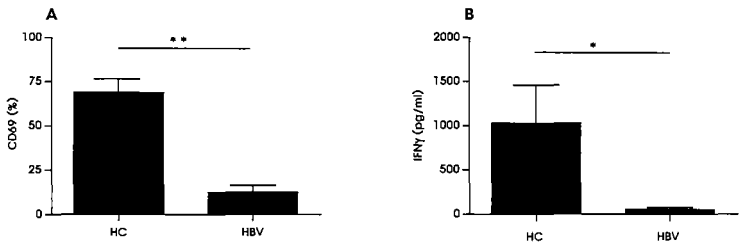
TABLE 1. CHARACTERISTICS OF STUDY POPULATION

		Healthy controls (n=16)	**Chronic HBV cohort (n=39)**
Age, years [mean ± SEM]		41.9 ± 2.2	42.5 ± 1.7
Sex	Female/male (%)	4/12	4/35
Race	Caucasian	7	14
	Asian	3	5
	Mediterranean	4	9
	Other	2	11
ALT, IU/mL [median ± IQ range]	n.a.	74 (50-101)	
HBV-DNA mean ^{10}log IU/mL ± SEM	n.a.	7.3 ± 0.3	
HBeAg	Positive/negative	n.a.	10/29
HBV genotype	Genotype A/B/C/D/E	n.a.	13/3/4/14/2
Fibrosis score (Metavir)	F0/F1/F2/F3/F4		2/14/11/9/3
Prior antiviral therapy	none	16	15
	NA		2
	(PEG) IFN		15
	(PEG) IFN + NA		7

Abbreviations: SEM; standard error of mean, IQ; interquartile, n.a.; not applicable, NA; nucleos(t)ide-analogues, (PEG)IFN; (pegylated) interferon-alpha

NK cell co-cultures of patients were strongly diminished as compared to controls (18 fold less; FIGURE 1B). There was no correlation between CD69 upregulation and IFN-γ production. Thus, in contrast to cells from HC, co-culture of polyI:C/IFNγ-activated mDC and NK cells from CHB patients results in only weak activation of NK cells, and poor cytokine production.

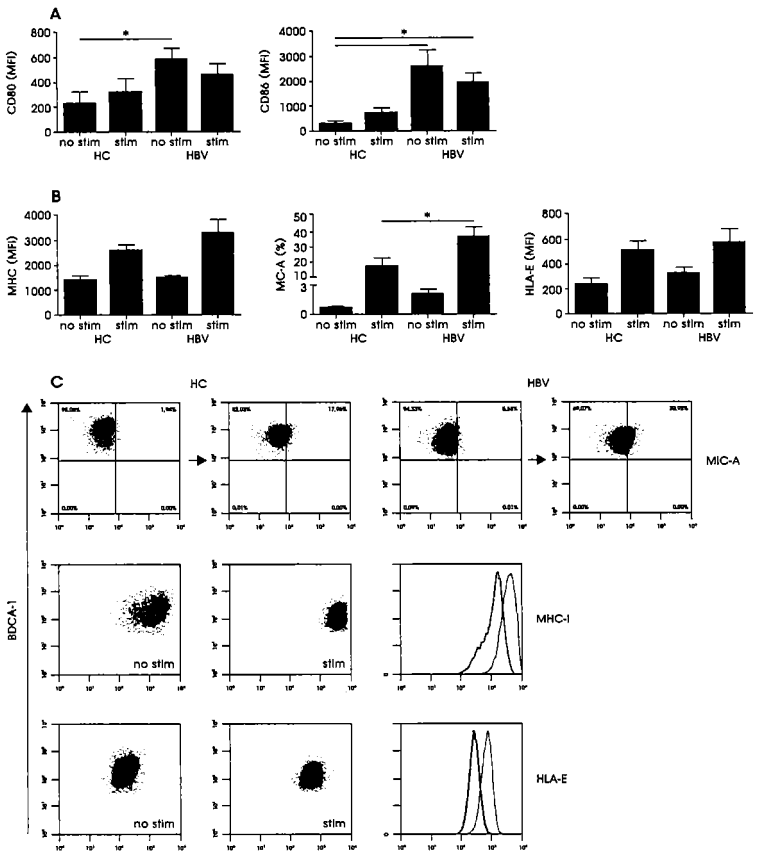
FIGURE 1. DIMINISHED mDC-INDUCED ACTIVATION AND FUNCTION OF NK CELLS IN CHB



Purified fresh NK cells were co-cultured with stimulated BDCA1+ mDC as is described in the Materials and Methods section. Cells were isolated from 11 healthy controls (HC) and 22 CHB patients (HBV) (mean ± SEM HBV-DNA ^{10}log 8.1±0.4 IU/ml, ALT 77±7 IU/mL). After 48h, the early activation marker CD69 on NK cells was determined by FACS analysis and supernatants of these cultures were evaluated for IFN-γ content by ELISA. Data are expressed as mean±SEM, and represent CD69 expressing NK cells within the total NK cell population (A) or IFN-γ levels in supernatant (B). *p<0.05, **p<0.01.

EXPRESSION OF MARKERS OF mDC MATURATION, BUT NOT OF NK CELL ASSOCIATED LIGAND EXPRESSION, ARE ENHANCED ON mDC OF CHB PATIENTS. To investigate if the activation and function of NK cells is impaired as a result of a disturbed interaction with mDC, we examined the response of mDC obtained from HC and CHB patients to stimulation with polyI:C and IFN-γ. As presented in FIGURE 2A, directly after isolation, the expression of the co-stimulatory molecules CD80 and CD86 on mDC was significantly higher in patients as compared to controls, indicating a higher maturation state of mDC ex vivo. Following stimulation, the expression of CD80 and CD86 weakly decreased in patients, but remained higher than the expression levels observed in HC. No correlation could be determined between CD80/CD86 expression and HBV-DNA or ALT levels (data not shown).

FIGURE 2. EXPRESSION OF MARKERS OF MATURATION ARE ENHANCED ON STIMULATED mDC OF CHB PATIENTS

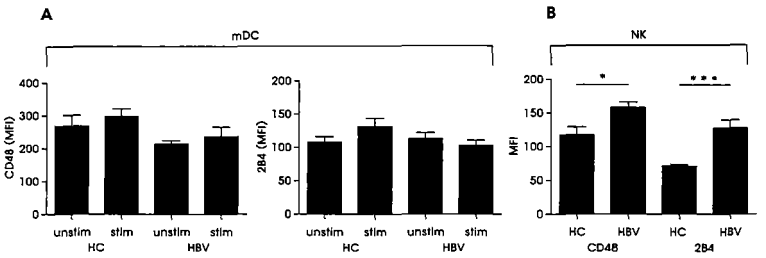


Isolated BDCA1+ mDC were stimulated with polyI:C and IFN-γ for 18h. Cells were isolated from 11 healthy controls (HC) and 22 CHB patients (HBV). Expression of CD80, CD86, MHC class I molecules (HLA-A, HLA-B, HLA-C), MIC-A and HLA-E were determined without stimulation (*no stim*) and after 18h stimulation with polyI:C and IFN-γ (*stim*). Expression levels of maturation markers CD80 and CD86 (A), and expression levels and percentage of cells positive for ligands MHC-I, MIC-A, HLA-E (B) and representive dotplots and histograms (C) as determined by flow cytometry are presented as mean±SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.0001$

To further characterize membrane-bound ligands on mDC for which cognate receptors are expressed on NK cells, we evaluated the expression of HLA-A/HLA-B/ HLA-C (indicated as "MHC-I"), MIC-A and HLA-E prior to and after stimulation with polyI:C and IFN-γ. As shown in FIGURE 2B, stimulation of mDC resulted in upregulation of the expression of MHC-I, MIC-A and HLA-E, which has been reported before[29]. Interestingly, the percentage of MIC-A expressing mDC upon stimulation was significantly higher in CHB patients as compared to HC. However, no difference in the percentage or MFI-levels between patients and controls could be demonstrated for MHC-I or HLA-E on stimulated mDC. Representative dotplots and/or histograms of the flow cytometric analysis are shown in FIGURE 2C. Thus, mDC from CHB patients showed a more activated phenotype accompanied by higher MIC-A expression, while the MHC-I and HLA-E molecules are not affected, which suggests limited involvement of these ligands in causing the impaired NK cell function in patients. Another molecule-pair that may affect the outcome of the interaction between mDC and NK cells is 2B4-CD48 (2, 7, 33). We evaluated the expression of CD48 and 2B4 on unstimulated and stimulated mDC (FIGURE 3A). No changes in the expression of CD48 and 2B4 on mDC were observed as a consequence of activation, and also the levels were similar between CHB patients and HC. In contrast, the expression of CD48 as well as 2B4 was significantly enhanced on NK cells freshly obtained from patients as compared to controls (FIGURE 3B). In this, the upregulation of CD48-2B4 on NK cells is not paralleled by upregulation on mDC which suggests that a major role for this receptor-ligand pair in mDC-NK cell interaction is unlikely since IFN-γ production by NK cells would require contact-dependent signals through 2B4 interaction[5].

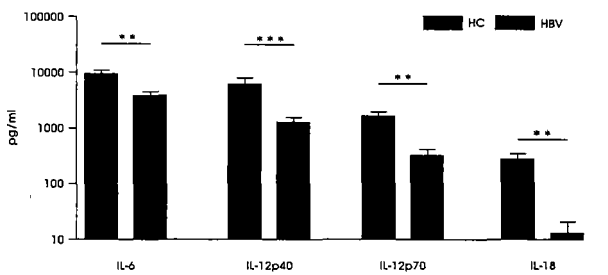
IMPAIRED CYTOKINE PRODUCTION BY mDC OF CHB PATIENTS. Besides contact dependent signals, also mDC-derived soluble factors, such as IL-12 and IL-18, are involved in mDC-NK cell interaction, and may activate NK cells[24]. After 18h activation with polyI:C and IFN-γ, supernatants of mDC of CHB patients contained less IL-6, IL-12 (IL-12p40 and IL-12p70) and IL-18 than HC (FIGURE 4). Therefore, the reduced capacity of mDC to produce cytokines may have consequences for the ability to activate NK cells in CHB patients.

FIGURE 3. EXPRESSION OF CD48 AND 2B4 ON MDC AND NK CELLS OF CHB PATIENTS



NK cells and mDC were purified from 5 healthy controls (HC) and 17 CHB patients (HBV) (HBV-DNA ^{10}log 6.3±0.5 IU/ml, ALT 96±17 IU/mL), and mDC were stimulated with polyI:C and IFN-γ for 18h. Expression levels of CD48 and 2B4 were determined on mDC without stimulation (*unstim*) and after 18h stimulation with polyI:C and IFN-γ (*stim*) and on NK cells. Expression levels of CD48 and 2B4 on mDC (A) and NK cells (B) by FACS are presented as mean±SEM. * $p<0.05$, *** $p<0.001$

FIGURE 4. IMPAIRED CYTOKINE PRODUCTION UPON STIMULATION OF mDC OF CHB PATIENTS



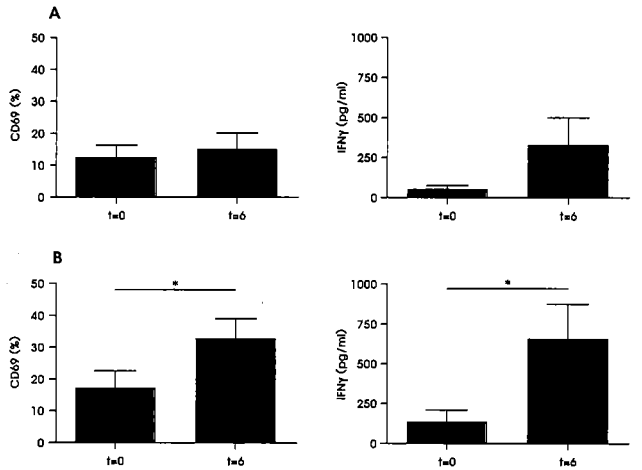
Purified mDC isolated from 5 healthy controls (HC) and 17 CHB patients (HBV) were stimulated with polyI:C and IFN-γ for 18h. Supernatants of these cultures were evaluated by ELISA or FACS (labelled beads). Data represent mean concentration (pg/ml) ± SEM of IL-6, IL-12p40, IL-12p70, and IL-18. ** $p<0.01$, *** $p<0.001$

ANTIVIRAL THERAPY AMELIORATED NK CELL FUNCTION AS A RESULT OF IMPROVED mDC-NK CELL INTERACTION. The effect of antiviral therapy on mDC-NK cell interaction and the capacity to produce antiviral cytokines was examined in CHB patients treated with entecavir, which is a nucleoside analogue that inhibits viral replication without direct effects on the immune system. All patients responded to therapy, resulting in a mean ^{10}log 4.2 IU/ml HBV-DNA reduction and mean 1.8-fold lowering of ALT levels after 6 months of treatment. After 6 months antiviral therapy, mean viral load±SEM was ^{10}log 3.1±0.3 IU/ml, mean ALT 36 IU/ml (range 25-56) and three HBeAg positive CHB patients achieved e-seroconversion. There was no difference in response between genotype, race and prior therapy, however groups were too small to reach significant differences (data not shown).

As shown in FIGURE 5A, as a consequence of antiviral therapy, the ability of mDC to activate NK cells, as demonstrated by CD69 expression, was not affected in co-cultures with synchronously isolated NK cells. In contrast, albeit not-significant, NK cell derived IFN-γ production was elevated during antiviral therapy in these cultures. To dissect the influence of persistent viral infection on the functionality of mDC and NK cells mDC isolated at baseline and isolated after 6 months of antiviral therapy, were co-cultured with autologous NK cells that were isolated 2 weeks prior to the start of therapy (FIGURE 5B). In comparison to their counterparts at baseline, mDC isolated during antiviral therapy were able to activate NK cells significantly better, which was paralleled by a significantly enhanced IFN-γ production. Since purified NK cells stimulated with IL-2 showed similar CD69 expression levels and IFN-γ production at baseline and during antiviral therapy (data not shown), it is likely that impaired mDC activation rather than an intrinsic NK cell defect, causes the reduced NK cell derived IFN-γ production during the interaction.

DECREASED EXPRESSION OF CD80, CD86 AND HLA-E DURING ANTIVIRAL THERAPY. Next, we investigated whether antiviral therapy results in altered NK cell induced mDC maturation. Prior to co-culture, the expression of CD80 and CD86 on freshly isolated as well as on activated mDC was not different irrespective of viral load reduction (data

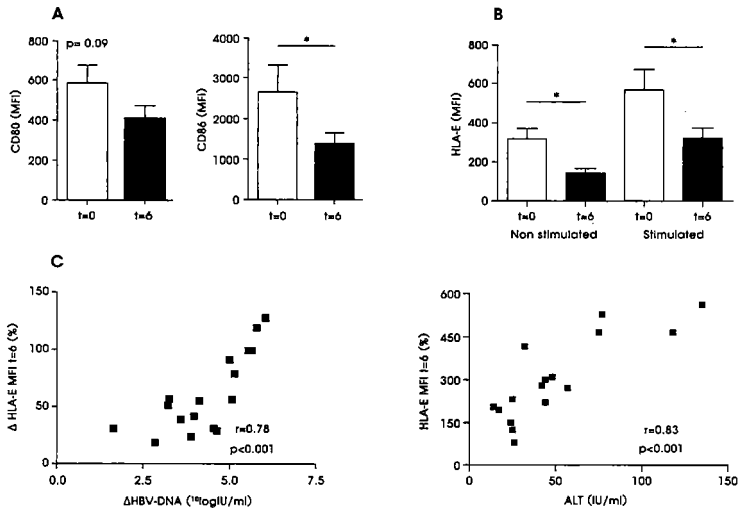
FIGURE 5. ANTIVIRAL THERAPY AMELIORATED NK CELL FUNCTION AS A RESULT OF IMPROVED mDC-NK CELL INTERACTION



Isolated NK cells were co-cultured for 48h with isolated BDCA1+ mDC after stimulation with polyI:C and IFN-γ for 18h. Cells were isolated from 22 CHB patients (HBV) at baseline (t=0) and after 6 months of treatment with entecavir 0.5 mg o.i.d. (t=6). (A) Frequency of fresh (A) or thawed (B) CD69-positive NK cells after 48h co-culture with simultaneously isolated mDC was determined at t=0 and t=6 by FACS analysis. Fresh NK cells were isolated simultaneous with mDC at t=0 and t=6. Thawed NK cells were isolated two weeks prior to baseline and frozen at -80°C before use. Supernatants of these cultures were evaluated for IFN-γ content by ELISA. Data represent CD69 expressing NK cells within the total NK cell population or mean levels of IFN-γ ± SEM. *$p<0.05$

not shown). In contrast, NK cell induced CD80 and CD86 expression was downregulated after 6 months antiviral therapy (FIGURE 6A). No correlation between CD80/CD86 expression and HBV-DNA or ALT levels could be found (data not shown). Upon antiviral therapy, the improved mDC-induced NK cell activation and production is thus paralleled by a lower NK cell-induced expression level of maturation markers on mDC. To investigate whether antiviral therapy also affected receptor/ligand expression, we determined the expression of MHC-I, MIC-A, HLA-E, CD48 and 2B4 on mDC and NK cells. Only the expression of HLA-E on

FIGURE 6. DIMINISHED ACTIVATION AND DECREASED EXPRESSION OF HLA-E ON mDC UPON ANTIVIRAL THERAPY



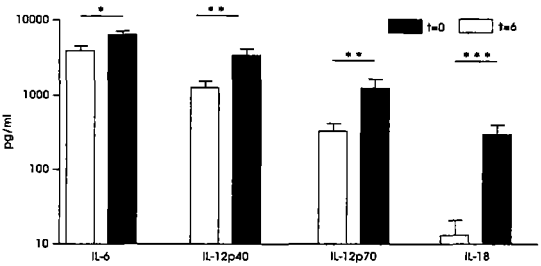
Purified fresh NK cells and mDC were isolated from 11 healthy controls (HC) and 22 CHB patients at baseline (t=0) and after 6 months of treatment (t=6). Expression of CD80 and CD86 on mDC upon co-culture with simultaneously isolated NK cells were determined by FACS analysis. Expression of HLA-E at t=0 and t=6 was determined on mDC without stimulation (*no stim*) and after 18h stimulation with polyI:C and IFN-γ (*stim*). Data represent mean ± SEM levels of expression of CD80 and CD86 (A) and HLA-E (B) on mDC. *p<0.05. (C) Spearman's rank correlation coefficient between the change in HBV-DNA (^{10}log IU/mL) and the change in mean fluorescence intensity (MFI) of HLA-E expression on mDC after stimulation at t=0 and t=6 was determined. Change in HBV DNA was defined as the absolute difference between levels at baseline and at 6 months. Change in MFI was defined as the percentage difference between levels at baseline and at 6 months. Spearman's rank correlation coefficient between the MFI of HLA-E expression on mDC after stimulation and levels of ALT (IU/ml) at t=6 was determined.

non stimulated and stimulated mDC, was significantly downregulated (FIGURE 6B), whereas the expression of all other molecules remained unchanged (data not shown). There was a strong correlation between HBV-DNA load reduction and the percentage downregulated HLA-E

expression on activated mDC (FIGURE 6C). Furthermore we observed an association between ALT levels during antiviral therapy and HLA-E expression (FIGURE 6C). The improved mDC-NK cell interaction may thus involve, at least partly, the expression of inhibitory receptor/ligand pairs which regulate the NK cell activation state.

ANTIVIRAL THERAPY ENHANCES MDC-DERIVED CYTOKINE PRODUCTION. To examine the effect of antiviral therapy on the capacity of mDC to produce cytokines, we determined IL-6, IL-12p40, IL-12p70 and IL-18 levels in supernatants of cultures at t=0 and t=6. Antiviral therapy resulted in significantly increased capacity of mDC to produce these cytokines (FIGURE 7).

FIGURE 7. ANTIVIRAL THERAPY AMELIORATES CYTOKINE PRODUCTION UPON STIMULATION



Purified mDC isolated from 17 CHB patients (HBV) were stimulated with poly I:C and IFN-γ for 18h at baseline (t=0) and after 6 months of treatment (t=6). Supernatants of these cultures were evaluated by immunoassay. Data represent mean concentration (pg/ml) ± SEM of IL-6, IL-12p40, IL-12p70, IL-18. *p<0.05, **p<0.01, ***p<0.001.

DISCUSSION

Bi-directional crosstalk between mDC and NK cells is known to play an important role in host immunity. In the present study, we examined the functional interaction between mDC and NK cells of CHB patients. We demonstrated that mDC of CHB patients are substantially impaired in their ability to activate NK cells, which, in turn, fail to secrete adequate amounts of IFN-γ. Expression of costimulatory molecules and to a lesser extent MIC-A were enhanced on polyI:C-stimulated mDC of CHB patients, while TLR-induced cytokine production by mDC was reduced. On NK cells of CHB patients the expression of CD48 and 2B4 was enhanced. Importantly, antiviral therapy with nucleos(t)ide-analogues improved mDC function, and we showed that this resulted in ameliorated mDC-induced NK cell activation and IFN-γ production. This was paralleled by decreased expression of CD80 and CD86 on mDC and HLA-E on NK cells, while TLR-induced cytokine production by mDC was enhanced. These data could not only partially explain the impaired NK cell activity upon chronic HBV infection, but may also provide a mechanism for an immunomodulatory role of antiviral drugs.

In unfractionated PBMC, we and others[26, 32] demonstrated improved NK cell function upon therapy. This may be attributed to the direct effect of viral load reduction on NK cells, however, our study provides evidence that mDC are at least partly responsible for the improved NK cell activation and function. NK cell activation is partly dependent on the balance between inhibitory and activating signals that are generated by an array of different cell-surface receptors after engagement by their specific cellular ligands[22]. We previously showed that NKp30 expression is significantly reduced in NK cells of CHB patients[32], which may compromise the mDC-NK cell interaction in which this activating receptor plays a critical role[15]. In HCV infection, HLA-E mediated signalling partly determines activation or inhibition of NK cell function[17]. Since expression of both HLA-E on mDC, as is demonstrated in this study, and its receptor NKG2A on NK cells decreases after antiviral therapy in CHB[32], we speculate that increased NK cell activation may be the result of this receptor/ligand pair. In the presence of a neutralizing antibodies against

NKG2A, CD69 expression on NK cells as the result of co-culture with mDC was indeed markedly increased (data not shown). However, the cytokine production was not affected, which indicated that NKG2A is not exclusively responsible for IFNγ production. As was previously described[31], we also observed an increase of the level of expression of 2B4 on NK cells of CHB patients. High-level 2B4 expression co-incided with an increased expression of PD-1 and a decreased HBV-specific proliferation and cytotoxicity of CD8+ T-cells[29]. Antiviral therapy did however not result in decreased expression of 2B4, limiting the role of this receptor/ligand pair in improved mDC-NK cell interaction. In addition to the above-mentioned regulatory molecules, Peppa et al. recently showed that blocking IL-10 restores NK cell antiviral function in HBV[26], and therefore an alternative explanation for the reduced NK cell activity may be mediated by DC-derived anti-inflammatory cytokines or additional negative regulation via inhibitory receptors[5].

Furthermore, we observed enhanced expression of CD80 and CD86 on mDC of CHB patients, which was reversed upon antiviral therapy. NK cell induced mDC show a strong ability to produce IL-12p70 and thus direct the differentiation of $CD4^+$ T cells and antigen-specific $CD8^+$ T cell responses[19]. In unfractionated PBMC of CHB patients, in parallel with the restored allostimulatory capacity of mDC upon antiviral therapy, an increased capacity to produce IL-12 was indeed observed[34]. As was suggested before[4], restoration of the reciprocal interaction between NK cells and mDC upon successful antiviral therapy, exemplified by an NK cell-derived IFNγ burst may lead to enhanced IL-12 production by activated mDC, which may trigger the adaptive immunity to overcome viral immune evasion[6]. In line with previous studies in CHB patients confirming functional impairment of mDC upon stimulation with cytokines and/or TLR ligands[11, 35, 21] this study shows lower cytokine production by mDC from patients upon stimulation with polyI:C and IFN-γ as compared to controls. Whether the mDC-induced NK cell function in CHB is also impaired upon the use of other stimuli remains to be resolved since the low number of circulating mDC and the limited volume of blood that can be withdrawn from the patients did not allow additional experimental conditions.

TLR3/mda-5 triggering with polyI:C may not exactly mimic the in vivo situation in CHB, but so far the physiological trigger for mDC activation has not been identified in CHB. However, this agonist is known to activate different mDC subsets, including the BDCA1+ mDC studied here, and proved to be suitable to examine the functional interaction between mDC and NK cells in both human and mice[16, 27, 15].

TLR-3 expression *in vivo* on BDCA1+ mDC has not been reported to be different in CHB patients. However, a decreased TLR3 protein expression in in vitro-generated monocyte-derived DC derived from CHB patients has been observed[18]. Since polyI:C induced TLR3 signalling in mDC is shown to be mandatory for production of IFN-γ by NK cells through various mechanisms involving the TRIF-inducing membrane protein INAM and RIG-1 receptor[15, 27], the reduced polyI:C-induced cytokine production may underlie the impaired DC-induced NK cell function.

In summary, on the basis of our findings obtained from in vitro co-culture experiments, we conclude that impaired NK cell function in chronic HBV patients may be partially be the result of HLA-restriction and decreased mDC-derived cytokine mediated activation. Additional blocking experiments may provide more evidence to elucidate the exact mechanism of the defective interaction. Upon antiviral therapy, the result of improved mDC function is enhanced mDC-NK cell interaction and consequently improved NK cell function.

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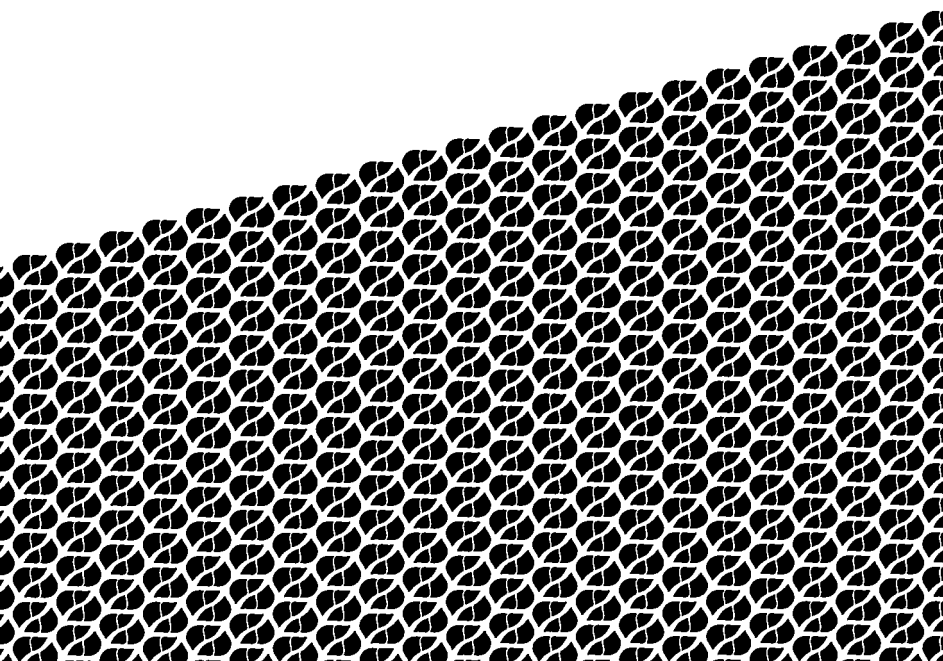

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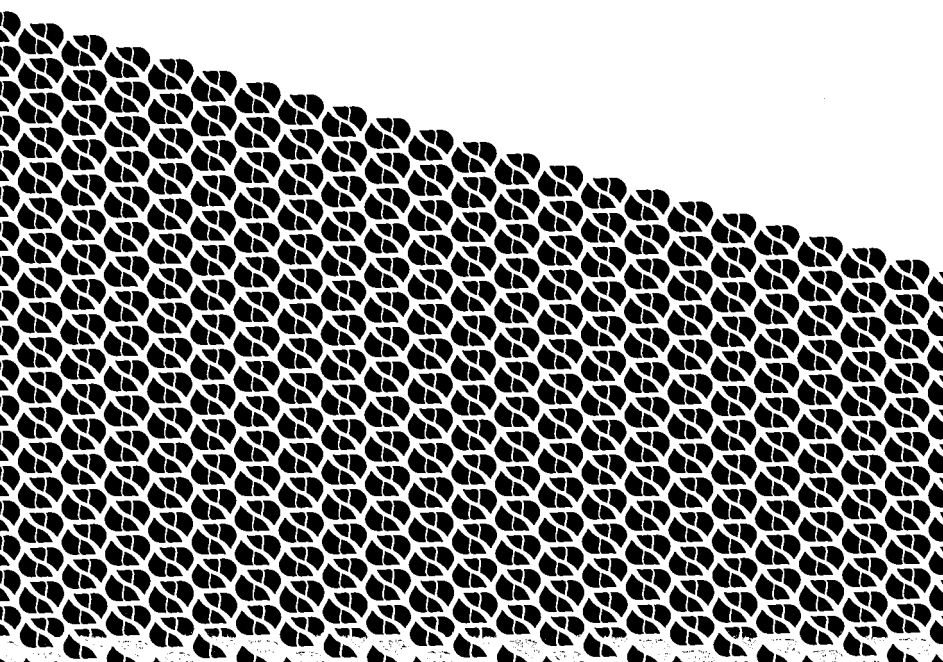


CHAPTER 5

INTRAHEPATIC NATURAL KILLER CELL ACTIVATION, BUT NOT FUNCTION, IS ASSOCIATED WITH $HBsAg$ LEVELS IN PATIENTS WITH $HBeAg$ NEGATIVE CHRONIC HEPATITIS B

ABSTRACT

BACKGROUND Natural killer (NK) cells play a major role in the regulation of anti-viral immunity. Since the hepatitis B virus (HBV) replicates in hepatocytes, examination of the liver of chronic hepatitis B (CHB) patients it is crucial to better understand the role of NK cells in HBV. HBeAg negative CHB differs in many aspects from HBeAg positive and until now little is known about the intrahepatic NK cell response in HBeAg negative patients. Intrahepatic immune control might be different in HBeAg negative as compared to HBeAg positive patients.
METHODS Liver NK cells were investigated in 21 HBeAg positive and 35 HBeAg negative CHB patients. Biopsy specimens were processed for routine histopathology and staging according to Ishak scores. Intrahepatic and blood NK cell frequencies, activation status and function of NK cells were analysed.
RESULTS In HBeAg negative CHB patients, compared to blood, liver NK cells displayed a more activated phenotype and stimulation further increased the activation status, but production of IFN-γ was markedly less. There was no difference with HBeAg positive CHB. Only in HBeAg negative CHB, but not in HBeAg positive CHB, NK cell activation was inversely correlated with the levels of HBsAg.
CONCLUSIONS The present study strongly suggests that liver NK cells of HBeAg negative CHB have a higher activation status compared to blood. However, they are not capable to increase cytokine production above levels reached by activated blood NK cells. Levels of HBsAg may contribute to the incapacity of activated liver NK cells to increase cytokine production in HBeAg negative CHB.

INTRODUCTION

Chronic hepatitis B (CHB) is the result of an inadequate immune response towards the virus[1]. The lack of a coordinated and efficient T cell response against HBV is underlying the viral persistence, but the mechanism behind this *failure* has not been elucidated[2-4]. HBV exclusively replicates within hepatocytes and the presence of covalently closed circular (ccc) HBV-DNA in the liver is the hallmark of viral persistence[5]. As a consequence, the development of an effective anti-HBV therapy leading to definite clearance requires insight in the mechanisms of HBV persistence in liver. Natural killer (NK) cells comprise almost one third of the intrahepatic lymphocyte population and exhibit potent antiviral activity[6, 7]. There is convincing data that blood NK cells are functionally impaired in CHB[8, 9, 10]. However, the phenotypical and functional characteristics of intrahepatic NK cells in CHB are less defined. It has been shown that flares during the natural course of CHB are the result of NK cell mediated apoptosis of infected hepatocytes[11]. Also in HCV, liver NK cells may be responsible for the liver damage without viral clearance[12].

The clinical phase of CHB is defined according to HBeAg status and ALT levels into immunotolerant (HBeAg positive, ALT normal), immunoactive (HBeAg positive, ALT raised), low replicative (HBeAg negative, ALT normal) and HBeAg negative CHB (ALT raised)[13]. Previous studies have examined the intrahepatic NK cell response during CHB in patient cohorts regardless of the clinical phase of CHB patients[14, 15, 16, 17]. Subgrouping based on HBV-DNA or ALT levels only partially accounts for the intrinsic differences between HBeAg positive and negative CHB. HBeAg negative patients comprise a subgroup of carriers of a non-replicative virus and having achieved e-seroconversion suggesting some immune control[18]. Nevertheless, also CHB patients infected with non-wild type HBV harboring pre-core mutants are included. In contrast to HBeAg positive patients, HBeAg negative patients are hard to treat with immunomodulatory therapy[19], which might be the result of difference in the quality of intrahepatic immune response. In contrast to HBeAg positive patients where activated NK cells were reported to accumulate in liver[20], little is known about the quality and function of liver NK cells in HBeAg negative CHB patients.

PATIENTS AND METHODS

PATIENTS AND ASSESSMENT OF VIROLOGICAL PARAMETERS. Liver biopsy specimens and peripheral heparinized blood samples were obtained simultaneously from 56 patients with chronic hepatitis B for cross-sectional analysis. All patients were negative for antibodies against hepatitis C, hepatitis D and human immunodeficiency virus. None of patients received any form of anti-viral treatment 30 days prior to biopsy. All biopsies were evaluated by two blinded pathologists and scored for periportal or periseptal interface hepatitis, portal inflammation and graded according to the Ishak scoring system. Fibrosis was also graded according to the Metavir scoring system.

HBV-DNA quantification was performed using COBAS Ampliprep/COBAS TaqMan test v02 (Roche; lower limit of quantification 20 IU/mL). HBV genotype was assessed using the INNO-LiPA assay (Innogenetics, USA). Serum HBsAg was quantified in samples using the ARCHITECT HBsAg assay (Abbott Laboratories; range 0.05-250 IU/mL). ALT was measured locally by standard procedures. The study was approved by the local ethics committee, and all patients gave written informed consent before liver biopsy and blood donation.

LIVER AND PERIPHERAL BLOOD NK CELL ENUMERATION. Liver biopsy specimens were obtained by standard protocols under ultrasonographic guidance. Biopsies were taken twice with a TruCut needle 14G and processed in formaldehyde for regular histopathologic evaluation. A distinct section of the biopsy (>5mm) was directly transferred to culture medium for further processing. Specimens were processed through a filter (70µm, BD, USA) and stained for FACS analysis. Simultaneously obtained whole blood samples were treated with ammonium chloride solution to lyse erythrocytes. To determine the percentage of NK cells, incubation with a cocktail of phycoerythrin (PE)-conjugated CD56 (MY31, BD Bioscience, Belgium), Pacific Blue-conjugated CD3 (UCHT1, Biolegend, USA), and AmCyan-conjugated CD45 (2D1, BD Bioscience,

Belgium) was performed for 15 minutes. Non-hemapoietic cells were identified as being CD45-negative. Cells were analysed using a multi-colour flow cytometer (FACS Canto II) and Diva software (both BD Bioscience, Belgium).

EXPRESSION OF INTRACELLULAR AND CELL SURFACE MOLECULES BY FLOW CYTOMETRY. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Isopaque (GE Healthcare, Finland) gradient centrifugation. For phenotypic analysis, incubation with a cocktail of FITC, PE, PE-CY7 and allophycocyanin (APC)-conjugated monoclonal antibodies (mAbs) was performed. The mAbs directed against the following molecules were used: CD3 (UCHT1), HLA-DR (L243), CD69 (L78) from BD Bioscience, Belgium; CD56 (N901), NKp44 (Z231) from Beckman Coulter, USA. As controls, cells were stained with isotype-matched control antibodies, and analysed as described above. Mean fluorescence intensity (MFI) and/or percentage positive cells were determined.

CYTOKINE PRODUCTION BY INTRACELLULAR CYTOKINE STAINING. Cytokine production by NK cells present in PBMC or liver biopsy specimens were determined by stimulation with 100 ng/mL IL-18 (MBL International Corporation, Japan) plus 10 ng/mL IL-12 (Miltenyi Biotec, Germany) for 24h in 24-well plates. In other experiments with healthy control PBMC and/or isolated blood NK cells, 0.2, 1 and 5 µg/ml plasma-derived HBsAg, subtype *ay* (ARP, USA) was added to the cultures. After 21h stimulation, 10 µg/mL brefeldin A (Sigma Aldrich, USA) was added. Non-stimulated cells were treated similarly and used as controls. PBMC or liver NK cells were fixed in 2% formaldehyde. After washing, cells were permeabilised with 0.5% saponin (VWR, USA) after which incubation with FITC-conjugated antibodies against IFNγ (B27, BD Pharmingen, USA) was performed. Cells were also stained with mAbs specific for surface CD3, CD56, CD69, CD45 and detected by FACS. Results are expressed as percentage of cytokine producing cells within the NK cell population.

STATISTICAL ANALYSIS. ALT levels are expressed as values representing a ratio to the local upper limit of normal (xULN). HBV DNA and HBsAg levels were were logarithmically transformed for analysis. Data are expressed as mean percentage ± SEM, unless indicated otherwise. Data were analysed with SPSS version 15.0 (SPSS Inc., USA) and Prism 5.0 (GraphPad software, USA) using the students t-test or Chi-square test to compare variables between 2 independent groups and the Spearman's rank correlation cefficient test for non-parametric correlations. In all analyses, a two-tailed P-valueof less than 0.05 (confidence interval 95% was considered statitically significant.

RESULTS

PATIENT CHARACTERISTICS. Phenotypic analysis of intrahepatic NK cells from paired liver biopsy and peripheral blood of 56 CHB patients was conducted. The patient characteristics are presented in TABLE 1. Twenty-one HBeAg positive and thirty-five HBeAg negative patients were comparable except for quantitative levels of HBV-DNA and HBsAg, which were higher in HBeAg positive patients. Levels of ALT, histological grading and staging according to Ishak scoring in HBeAg negative CHB were not different from HBeAg positive CHB.
Evaluation of serum parameters showed a strong correlation between HBV-DNA and HBsAg. This correlation was stronger in HBeAg positive than in HBeAg negative CHB patients (r-values 0.64 ($p<0.01$) vs 0.39 ($p<0.05$)). Grading of liver inflammation according to Ishak scoring, weakly correlated with HBV-DNA levels in HBeAg negative patients, and with ALT in HBeAg positive patients (r-value 0.39, $p<0.05$ resp 0.44, $p<0.05$). Staging of fibrosis did not show a correlation with ALT, HBV-DNA and HBsAg levels, both in HBeAg-positive and HBeAg-negative patients.

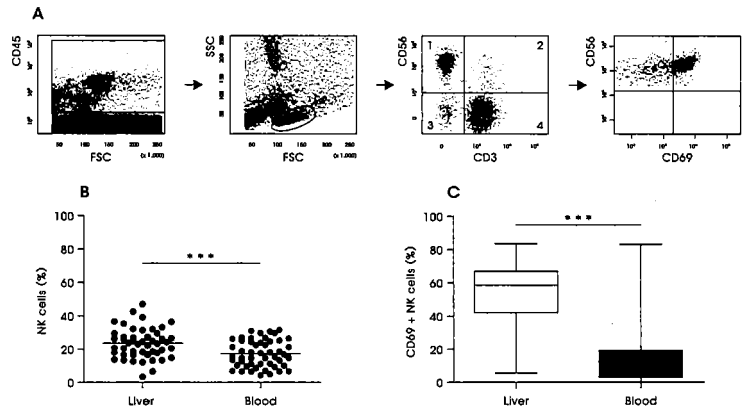
TABLE 1. CHARACTERISTICS OF STUDY POPULATION

		HBeAg-positive (n=21)	HBeAg-negative (n=35)	p-value
Age, years [mean ± SEM]		34.4 ± 2.1	39.9 ± 1.9	0.15
Female/male		11/10	14/21	0.37
ALT, IU/mL [median ± IQ range]		94.8 ± (35-145)	75.3 ± (37-92)	0.21
HBV-DNA mean ^{10}log IU/mL ± SEM		6.9 ± 0.5	5.1 ± 0.3	0.002
HBsAg IU/mL [mean ± SEM]		21403 ± 4443	6917 ± 1251	0.002
Genotype A/B/C/D/E		2/6/5/7/1	7/8/6/9/5	0.31
Ethnic	North European	6	7	
background	Asian	11	13	
	Mediterranean	2	10	
	Other	2	5	
Histology				
Ishak total [mean ± SEM]		7.1 ± 0.8	6.5 ± 0.6	0.61
Ishak grading total (mean ± SEM)		5.3 ± 0.6	4.5 ± 0.4	0.40
Ishak staging (mean ± SEM)		1.8 ± 0.3	1.8 ± 0.2	0.82

Abbreviations: SEM; standard error of mean, IQ; interquartile,

FREQUENCY AND ACTIVATION STATUS OF LIVER NK CELLS IN CHB PATIENTS. The contribution of CD3-CD56+ NK cells within the CD45+ leukocytes was determined in both liver and blood of CHB patients. Representative FACS plots of liver leukocytes are shown in FIGURE 1A. We observed a 1.4 fold higher frequency of NK cells in liver than in blood (FIGURE 1B). Next, we examined the activation status of NK cells and showed that the frequency of cells expressing the early activation marker CD69 is 3.3-fold higher in liver than in blood (FIGURE 1C). In line with CD69, the frequency of NK cells expressing other activation markers such as NKp44 is almost 8-fold higher in liver than in blood (17% vs 2%), and approximately one third of blood NK cells is positive for HLA-DR expression compared to more than half of liver NK cells (33% resp 50%). Furthermore, NK cells show similar activation profile in HBeAg negative disease compared to HBeAg positive disease.

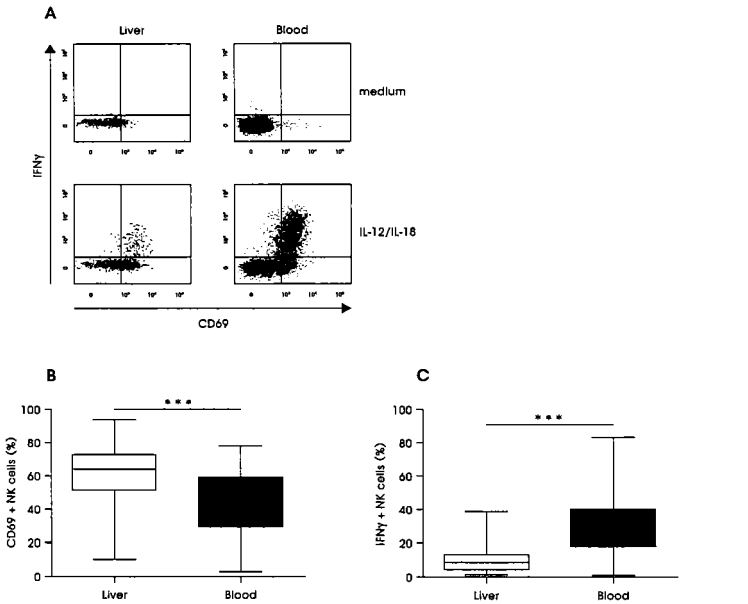
FIGURE 1. THE FREQUENCY AND ACTIVATION STATUS OF NK CELLS IS HIGHER IN THE LIVER THAN BLOOD OF CHB PATIENTS



The frequency of CD56+CD3- NK cells was determined by flow cytometry within the population of CD45+ leukocytes from liver biopsies and peripheral blood from 56 CHB patients. Representative FACS plots showing the gating strategy are depicted (A) Data shown represent the percentage of NK cells in each individual within the total lymphocyte population (B) and of NK cells positive for the early activation marker CD69 as shown in box-and-whisker plots (C). Data are presented as mean±SEM. *** *P<001.*

IN VITRO STIMULATION OF LIVER NK CELLS AND PRODUCTION OF IFNγ. As qualitative immunohistochemical analysis of liver tissue of HBeAg positive patients showed a marked increase of IL-12/18 positive intrahepatic cells[20] and IL-12/18 is a potent inducer of cytokine production by blood NK cells[21-22], we investigated whether the HBeAg status influences the cytokine production of activated liver NK cells. Stimulation of liver NK cells with IL-12/18 further augmented the baseline activation levels, and resulted in significantly more CD69 expressing cells compared to stimulated blood NK cells (FIGURE 2A and 2B). Interestingly, although liver NK cells were more activated, evaluation of the frequency of IFNγ-producing cells showed that the production of IFNγ was less by liver NK cells compared to blood NK cells (11% vs 30%, p<0.001) (FIGURE 2C). However, the finding that liver NK cells of HBeAg

FIGURE 2. ACTIVATION, BUT NOT INTRACELLULAR IFNγ POSITIVITY, OF NK CELLS IS HIGHER IN THE LIVER THAN BLOOD OF CHB PATIENTS

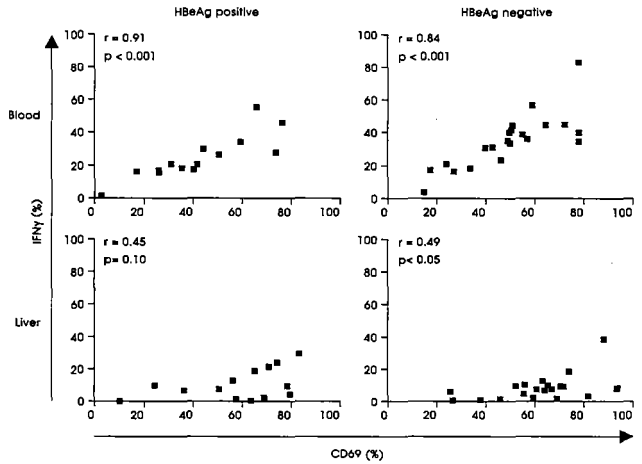


Activation status and intracellular staining for IFNγ upon 24h IL-12/18 stimulation of liver and PBMC were simultaneously determined in total NK cells from 14 HBeAg positive and 21 HBeAg negative patients. Representative FACS plots are depicted (A). Data shown represent the percentage of CD69 (B) and IFNγ expressing cells (C) in total NK cells, as shown in box-and-whisker plots. Data are presented as mean±SEM. **** P<001.*

negative patients show higher CD69 expression and lower frequencies of IFNγ-producing cells than blood NK cells upon activation is not different in HBeAg positive CHB (data not shown).

Interestingly, a strong correlation was observed between NK cell activation and cytokine production in blood NK cells (FIGURE 3) and this trend was also observed in liver NK cells. Importantly, these responses to IL-12/18 were similar between NK cells from HBeAg positive and HBeAg negative CHB (data not shown).

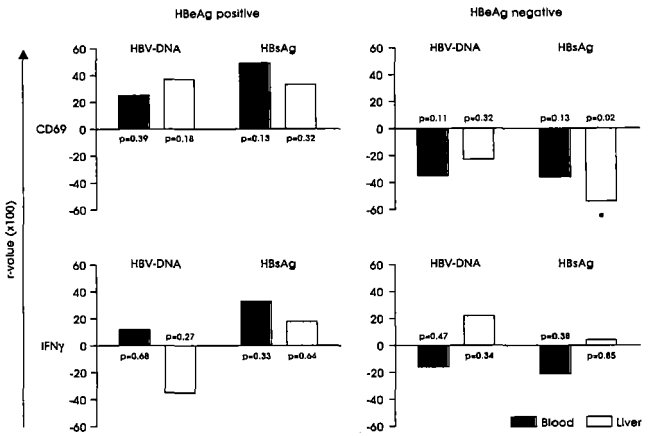
FIGURE 3. CORRELATION BETWEEN ACTIVATION STATUS AND INTRACELLULAR IFNγ IN NK CELLS IN THE LIVER AND BLOOD OF CHB PATIENTS



Activation status and intracellular staining for IFNγ upon 24h IL-12/18 stimulation of liver and PBMC was simultaneously determined in total NK cells from 14 HBeAg positive and 21 HBeAg negative patients. Spearman's rank correlation coefficients between the IL-12/18-induced CD69-positivity and IFNγ expression in NK cells in liver and blood were determined.

CORRELATION BETWEEN HBV-DNA OR HBsAg LEVELS AND LIVER NK CELL ACTIVATION OR CYTOKINE PRODUCTION. Since levels of HBsAg and to a lesser extent HBV-DNA are well profiled in HBeAg positive and negative CHB[25] and subsequently also significantly different between the two groups in our study, we investigated whether HBsAg and HBV-DNA levels influenced the activation status and cytokine profile of liver NK cells. Univariate correlation analysis was performed on the frequency of liver NK cells expressing CD69 upon IL-12/IL-18 stimulation and levels of HBsAg and HBV-DNA (FIGURE 4). Only in HBeAg negative patients, the induced expression of CD69 on liver NK cells – but not blood NK cells- inversely correlated with HBsAg levels (r=-0.57, p 0.02). In contrast, in HBeAg positive disease, induction of CD69 expression by liver NK cells did not correlate with the levels of HBV-DNA or HBsAg in serum.

FIGURE 4. CORRELATION BETWEEN ACTIVATION STATUS AND HBsAg IN NK CELLS IN THE LIVER AND BLOOD OF HBeAg NEGATIVE CHB PATIENTS



Activation status and intracellular staining for IFNγ upon 24h IL-12/18 stimulation of liver and PBMC was simultaneously determined in total NK cells from 14 HBeAg positive and 21 HBeAg negative patients. Spearman's rank correlation coefficients between the IL-12/18-induced CD69-positivity, IFNγ expression and levels of HBV-DNA and HBsAg in NK cells in liver and blood were determined. * P<0.05.

Regardless of the HBeAg status, the frequency of IFNγ-producing NK cells in both blood and liver did not correlate with the levels of HBsAg or HBV-DNA. Furthermore, also the stage of fibrosis, grade of inflammation and ALT levels did not correlate with activation or IFNγ production by liver NK cells (data not shown). Levels of HBsAg may thus influence the activation status of liver NK cells in HBeAg negative CHB, but not their capacity to produce antiviral cytokines.

DISCUSSION

The local immune response in the liver is important for the outcome of CHB and the persistence of HBV in the liver, since replication takes place in hepatocytes. Blood NK cells have an impaired ability to become activated and produce cytokines in CHB, but solid data on liver NK cells is currenlty lacking. In HBeAg positive CHB, liver NK cells were strongly activated and cytotoxic *in vitro*[29]. However, immune control mechanisms underlying HBeAg negative CHB may differ considering the presence of a non-replicative virus, serological profile of HBsAg levels and moderate treatment response on immunomodulatory therapy[24]. This is the first report on activaton status and function of liver NK cells in HBeAg negative CHB.

In our study, NK cells from HBeAg negative CHB patients displayed a more activated phenotype in liver than in blood. Stimulation with IL-12/18 further increased the activation status of the NK cell pool, but production of IFN-γ by NK cells was markedly less in liver than in blood. Only in HBeAg negative CHB, activation was inversely correlated with the levels of HBsAg suggesting distinct intrahepatic immunoregulatory mechanisms compared to HBeAg positive disease.

Murine and human *in vitro* experiments have shown that the immune response to HBV is differently induced by its principal antigens. For instance, HBeAg has been shown to induce a Th2 profile in mice, whereas HBcAg induced a Th1 response[25]. HBsAg, its levels notably distinctive between HBeAg positive and negative CHB, impairs components of the innate immune response hampering direct antiviral cytokine production and antigen presentation by different subsets of dendritic cells[26, 27, 28].

Intrahepatic NK cells of HBeAg negative patients are more activated as compared to blood NK cells as is shown by expression of CD69, HLA-DR and NKp44. Liver NK cells in CHB may become activated by IL-12, IL-15 and IL-18[29]. We show that IL-12/18 induced cytokine production was less in liver NK cells than in blood NK cells. Until now, this was not reported in HBeAg negative CHB patients. We now show that in HBeAg negative patients augmented CD69 expression and a reduced frequency of IFNγ-

producing NK cells was observed in the liver as compared to blood. Our findings differ from those reported by Zhang et al. in HBeAg positive CHB patients. In this study, higher frequencies of IFNγ-producing liver NK cells were observed upon stimulation with PMA and ionomycin as compared to blood NK cells[29]. The observed differences may be due to differences in immune status of patients mediated by, for instance, differential regulation by IL-10 and TGF-beta in HBeAg negative compared to positive CHB patients. However, also the stimulus may be relevant. We chose fysiological stimuli known to be expressed in the liver during HBV infection[29] and important in the crosstalk between key players of the innate immune response[22]. The enhanced baseline activation status of liver NK cells compared to blood may thus be the result of the presence of intrahepatic IL-12/18. Moreover, IL-12 induced STAT-4 phosphorylation was less in chronic HCV patients compared to controls *in vitro*[29] and similar processes may explain the hampered cytokine production in CHB. We also showed that activation of liver NK cells is inversely correlated with serum HBsAg in HBeAg negative CHB. HBsAg may modulate the intrahepatic NK cell compartment differently in HBeAg positive disease. Recent studies indicate that HBsAg levels specifically reflect the phase of CHB infection, and are lower in HBeAg negative CHB than in HBeAg positive CHB[23, 59]. Until now it is not known what the immunomodulatory effects of HBsAg are on liver NK cells. In contrast to HBeAg positive CHB, no association was found between HBsAg production and HBV replication via intrahepatic cccDNA in HBeAg negative CHB[31]. This loss of association could be explained by viral integration into the host genome and may affect immune control[59]. An explanation may concern the more profound immunomodulatory dynamics of NK cell activation by HBsAg in the ranges observed in HBeAg negative CHB in the absence of other viral proteins i.e. HBeAg compared to high HBsAg levels in HBeAg positive CHB. To gain insight in the role of HBsAg in NK cell activation and cytokine production, an *in vitro* proof-of-principle experiment was conducted in which healthy control PBMC and isolated blood NK cells were stimulated with IL-12/18 in the absence or presence of dose-ranged plasma-derived HBsAg. Stimulation of NK cells in the presence of HBsAg did not augment CD69 expression, and did not

affect IFNγ production by NK cells (data not shown). Albeit indirect, HBsAg may play a role in the intrahepatic NK cell response in HBeAg negative patients, but the exact relationship between HBeAg, HBsAg and regulatory cytokines, such as IL-10, needs further research. Also more detailed studies to explain the opposing consequences of chronic HBV infection on activation and cytokine production by intrahepatic and blood NK cells upon stimulation with specific specific stimuli need to be conducted.

In conclusion, the present study strongly suggests that liver NK cells of HBeAg negative CHB patients are more activated than in blood which corroborates earlier observations in HBeAg positive CHB. Since we observed that in HBeAg negative patients, intrahepatic NK cell activation negatively correlates with HBsAg levels, lowering these HBsAg levels may serve an important target for therapy aimed at improving intrahepatic immunity by innate immune cells.

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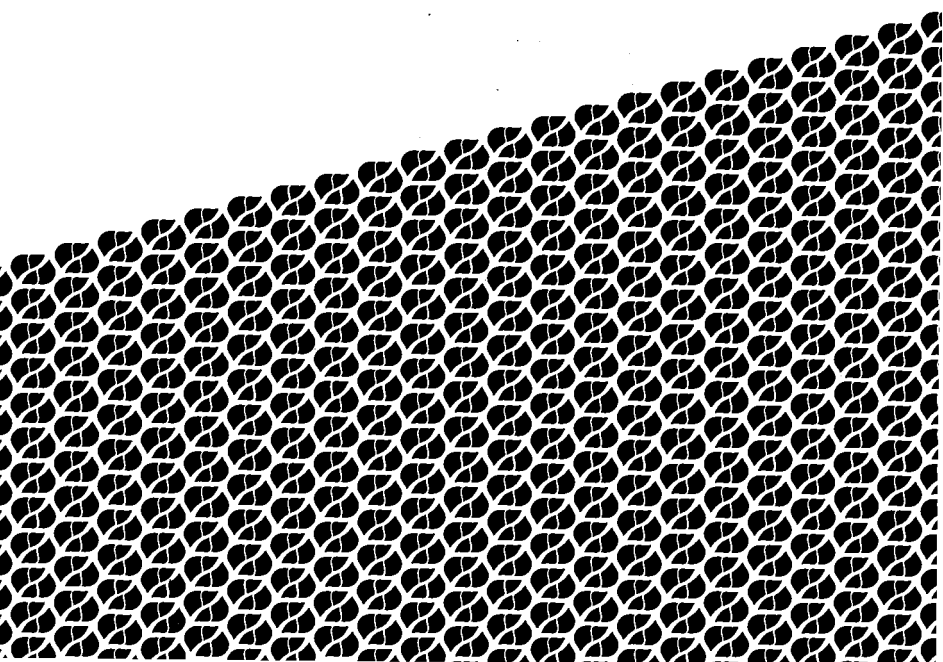

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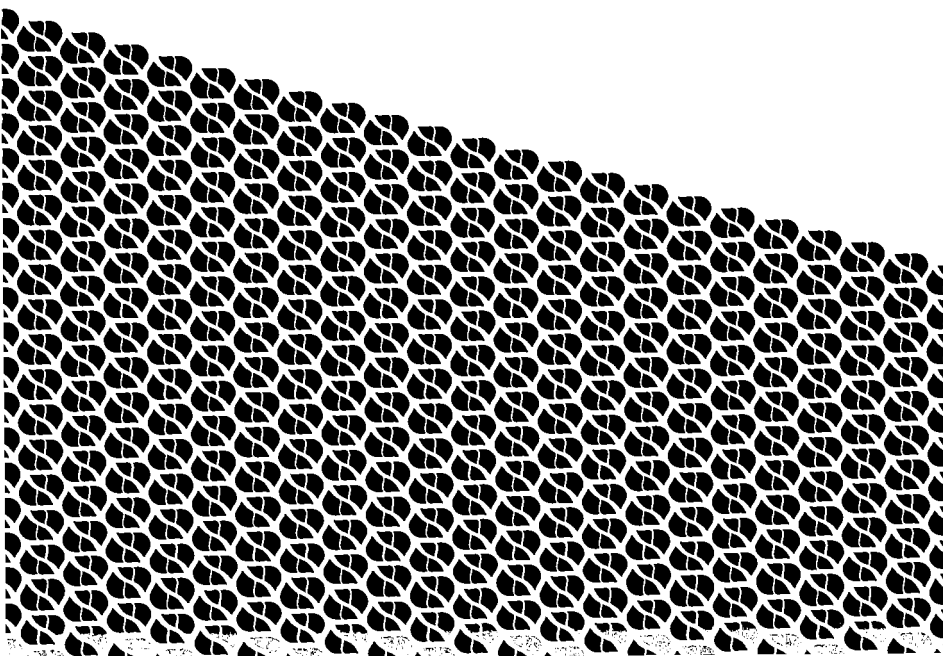


CHAPTER 6

TENOFOVIR TREATMENT OF CHRONIC HEPATITIS B PATIENTS HAS MINIMAL EFFECTS ON INTRAHEPATIC NK CELL FREQUENCY AND ACTIVATION STATUS

ABSTRACT

Natural killer (NK) cells have an impaired ability to become activated and produce cytokines in chronic hepatitis B (CHB). Antiviral therapy of CHB patients partially overcomes this defect in blood NK cells, but there are no reports on liver NK cells.
We show that activation of liver NK cells is associated with high baseline viral load conditions. Additionally, repetitive sampling during successful therapy with tenofovir showed no modulation of frequency and minor effects on activation status of liver NK cells. We conclude that activation of liver NK cells is not a pivotal determinant in antiviral treatment in CHB.

INTRODUCTION

Current treatment of chronic hepatitis B virus (CHB) consists of pegylated interferon-α (PEG-IFN) and/or nucleos(t)ide analogues (NUCs). There is limited data on the effects of treatment with NUCs on the immune response against HBV. The function of blood T-cells improved in NUC-treated CHB patients, but this effect was only transient[1]. Circulating NK cells obtained from CHB patients exerted normal cytotoxicity comparable to healthy individuals, but they were less activated and produced less IFN-γ[2-4]. The defective ability of NK cells to produce IFN-γ may have important consequences for the control of viral replication via its direct cytotoxic effects and ability to promote Th1 responses[5]. Since HBV replicates exclusively in hepatocytes, examination of the liver of CHB patients is crucial to better understand the role of NK cells in antiviral immunity to HBV. It was reported that the frequency of liver NK cells in CHB patients was lower than in healthy individuals[6], but higher than in patients with chronic HCV infections[7]. We and others recently showed partial restoration of the antiviral potential of blood NK cells following treatment of CHB with entecavir[4] or lamivudin and adefovir[5]. In blood, restoration of NK cell activity upon viral load

reduction, demonstrated 6 months after start of entecavir therapy, was accompanied by an enhanced number of IFN-γ-producing CD56+ NK cells as well as normalization of the expression of the activating receptor NKG2A on circulating NK cells. At present, no longitudinal studies have been conducted in CHB addressing the effect of viral load reduction on the intrahepatic NK cell compartment.

In the present study, for the first time multiple sampling of the liver of CHB patients during the course of tenofovir therapy was performed to determine the frequency, phenotype and activation status of liver NK cells. We observed that viral load decline did not strengthen the intrahepatic NK cell compartment in CHB patients.

PATIENTS AND METHODS

PATIENTS. Fine needle aspiration biopsy (FNAB) specimens and peripheral heparinized blood samples were obtained simultaneously from 11 patients with chronic hepatitis B prior and during treatment with the nucleotide analogue tenofovir dipivoxil (245 mg o.i.d.). Patients receiving anti-viral therapy within 6 months prior to treatment were excluded. All patients were negative for antibodies against hepatitis C, hepatitis D and human immunodeficiency virus. The study was approved by the local ethics committee, and all patients in the study gave informed consent before FNAB and blood donation.

LIVER AND PERIPHERAL BLOOD LYMPHOCYTE CELL ENUMERATION. Liver leukocyte specimens were obtained as was previously described[5] at baseline, week 12, 24 and 48 of antiviral therapy. To determine the percentage of NK cells, cells were incubated with CD56 (MY31, BD Bioscience, Belgium), CD3 (UCHT1, Biolegend, USA) and CD45 (2D1, BD Bioscience, Belgium). Stained cells were analysed using a multi-color flow cytometer (Canto II) and Diva software (both BD Bioscience, Belgium).

EXPRESSION OF INTRACELLULAR AND CELL SURFACE MOLECULES BY FACS. Antibodies against the following molecules were used: CD3 (UCHT1), HLA-DR (L243), CD69 (L78), CD25 (2A3) from BD Bioscience, Belgium; CD56 (N901), NKG2A (Z199), NKG2D (ON72) from Beckman Coulter, USA. As controls, cells were stained with corresponding isotype-matched control antibodies. Stained cells were analysed as described above. Mean fluorescence intensity (MFI) and/or percentage positive cells were determined.

STATISTICAL ANALYSIS. Data are expressed as mean percentage ± SEM, unless indicated otherwise. Data were analysed with Prism 5.0 (GraphPad software, USA) using the Mann-Whitney U test to compare variables between 2 independent groups and the Wilcoxon matched pairs test between paired variables. In all analyses, a two-tailed P-value of less than 0.05 (confidence interval 95%) was considered statistically significant.

RESULTS

To examine the effect of antiviral therapy on the intrahepatic NK cell compartment, we performed multiple aspirate liver biopsies from 11 CHB patients before and during tenofovir treatment (TABLE 1). From the total aspirate, on average 15,000 leukocytes were acquired by flow cytometry at each time point, which allowed us to perform flow cytometric analysis but no other assays. At week 48 of treatment, serum HBV DNA was undetectable in all patients, and 7 patients normalized ALT levels at week 48. All patients were still on tenofovir treatment at the end of the study. Six patients were classified as "high viral load" (HBV DNA>10^5 IU/mL) and five patients as "low viral load" (HBV DNA<10^5 IU/mL) prior to antiviral treatment.

THE FREQUENCIES OF INTRAHEPATIC AND CIRCULATING NK CELLS REMAIN STABLE UPON THERAPY. In liver, we observed a 1.5 fold higher frequency of NK cells than in blood (FIGURE 1A). The number of intrahepatic NK cels monitored by evaluating aspirate biopsies at

TABLE 1. CHARACTERISTICS OF STUDY POPULATION

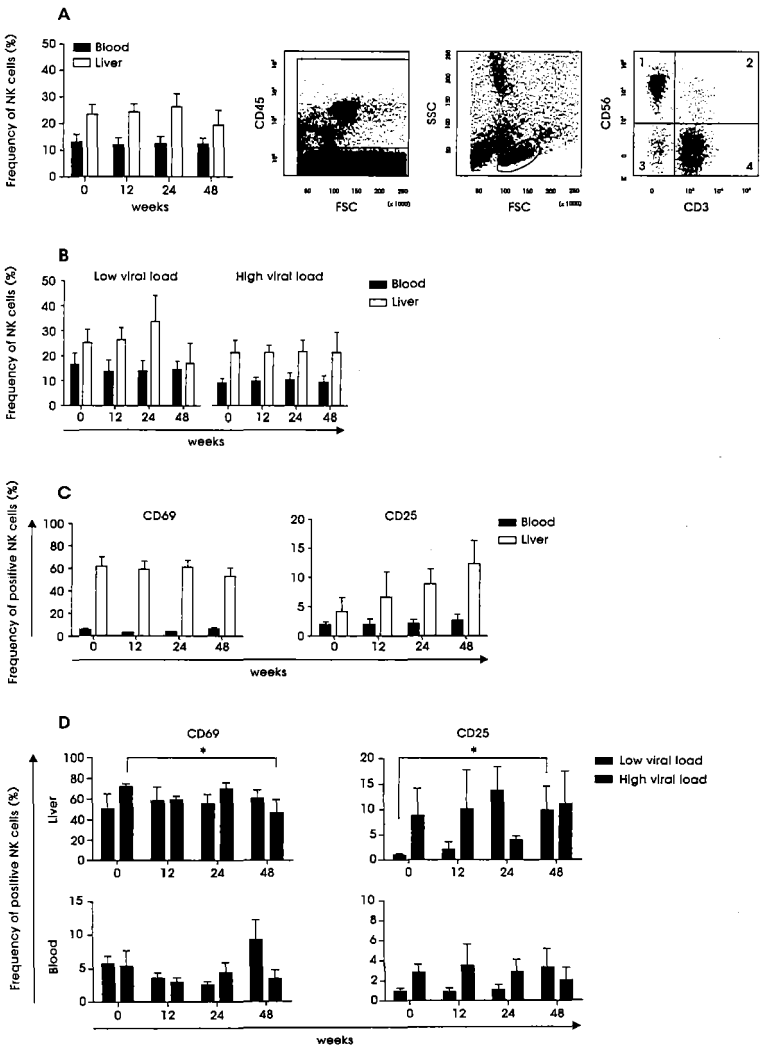
		Cohort (n=11)	High viral load (n=6)	Low viral load (n=5)
Age, years [mean ± SEM]		40.4 ± 1.4	43 ± 6.1	37.2 ± 5.9
Sex	Female/male (%)	1/10	0/6	1 / 4
Race	Caucasian	2	1	1
	Asian	3	2	1
	Mediterranean	4	3	1
	Other	2	0	2
ALT, IU/mL [median ± IQ range]		76 (33-96)	98 (46-168)	48 (23-69)
HBV-DNA mean log^{10} IU/mL ± SEM		5.4 ± 0.5	6.6 ± 0.5	4.1 ± 0.4
HBeAg Positive/negative		1/10	1/5	0/5
HBV genotype Genotype A/B/C/D/E		4/2/0/4/1	2/1/0/3/0	2/1/0/1/1

Abbreviations: SEM; standard error of mean, IQ; interquartile

4 different time-points, remained constant throughout the observation period. A weak reduction of liver NK cells was observed at week 48, but this was not significant. In agreement with our previously published findings[4], the number of blood NK cells did not change upon therapy. Also when baseline HBV DNA levels of patients were considered, we did not observe changes in the frequency of peripheral or intrahepatic NK cells in patients with a low or high viral load prior to start of treatment (FIGURE 1B).

THE ACTIVATION STATUS OF LIVER NK CELLS IS DISTINCT FROM BLOOD NK CELLS, BUT IS NOT CHANGED BY ANTIVIRAL THERAPY. Next, the activation status of intrahepatic NK cells from CHB patients was determined during the course of treatment as the percentage of liver NK cells expressing CD69 or CD25. Prior to treatment, the activation status of NK cells differed between liver and blood in patients (FIGURE 1C). The frequencies of NK cells expressing CD69 and CD25 were higher at baseline on intrahepatic NK cells as compared to circulating NK cells. Upon treatment, no modulation of the number of CD69 expressing liver NK cells were observed. Similarly, also on blood NK cells no modulation

FIGURE 1. THE FREQUENCY OF NK CELLS IS HIGHER IN THE LIVER THAN BLOOD OF CHB PATIENTS AND REMAINS STABLE THROUGHOUT TENOFOVIR TREATMENT. THE ACTIVATION STATUS OF LIVER NK CELLS IS DISTINCT FROM BLOOD NK CELLS, AND IS NOT CHANGED BY TENOFOVIR TREATMENT



(A) The frequency of CD56+CD3- NK cells was determined by flowcytometry within the population of CD45+ leukocytes from fine needle aspiration biopsies and peripheral blood from 11 CHB patients. A representative FACS plot showing the gating strategy is depicted. (B) The patient groups were further subdivided as 5 CHB patients with low viral load (HBV DNA<10^5 IU/mL) and 6 CHB patients with high viral load (HBV DNA>10^5 IU/mL). (C) The expression of CD69 and CD25 was determined by flow cytometry on CD56+CD3- NK cells in blood and liver aspirate biopsies from 11 CHB patients. (D) The data was also analyzed by comparing the frequencies, prior to therapy, of 5 patients with a low viral load and 6 CHB patients with a high viral load. Samples were analyzed at baseline, and at week 12, 24 and 48 of therapy with tenofovir 245 mg o.i.d.. Data are expressed as mean ± SEM. * denotes $p<0.05$.

of CD69 expression was observed during therapy. However, although the number of CD25 expressing blood NK cells was not affected, in the liver a significant increase was observed of the frequency of CD25 positive intrahepatic NK cells after 48 weeks of treatment.
As shown in FIGURE 1D, the percentage of intrahepatic NK cells that expressed CD69 at baseline was 1.4 fold higher in high viral load patients than in low viral load patients, which was not found for circulating NK cells. Upon treatment, the percentage of CD69 expressing liver NK cells was significantly decreased in only patients who had a high viral load prior to therapy, but not in patients with a relatively low viral load.
Finally, the frequency of CD25 expressing NK cells showed that CD25 expression was relatively low on both liver and blood NK cells. However, albeit not significant, high viral load patients had a 9 fold higher frequency of CD25 expressing NK cells in the livers than low viral load patients. At week 48 during the course of treatment, the number of CD25 expressing liver NK cells in low viral load patients was significantly increased and similar to the high viral load patients, in whom the number of CD25 expressing NK cells remained stable during therapy.

NKG2A AND NKG2D EXPRESSION ON LIVER NK CELLS IS NOT DEPENDENT OF VIRAL LOAD. At baseline the frequency of the inhibitory receptor NKG2A and the activating receptor NKG2D expressing NK cells was comparable in the liver and blood of patients. Antiviral therapy did not affect the expression and the frequency of NKG2A and NKG2D positive NK cells in CHB patients (data not shown).

DISCUSSION

The local immune response in the liver is important for the outcome of CHB and the persistence of HBV in the liver, since replication takes place in hepatocytes. Since the dynamics of the immune response in the livers of CHB patients during antiviral therapy is largely unknown, we monitored the intrahepatic NK cell compartment by collecting for the first time multiple fine needle aspirate biopsies during tenofovir therapy of CHB patients. Longitudinal evaluation of the liver showed that as a consequence of antiviral therapy, the frequency of intrahepatic NK cells, their activation status and the expression of NKG2A and NKG2D were not or only minimally affected. However, subtle differences were observed in the activation markers on intrahepatic NK cells in patients with variable HBV DNA levels. Our longitudinal analysis thus extends previous evaluations of intrahepatic immunity in CHB patients since these studies were cross-sectional and/or compared the intrahepatic NK cells between different patient groups[6, 7, 9-11]. In this unique study, we now show that tenofovir treatment of CHB patients does not increase or normalize liver NK cell frequencies. Recently, it was shown that tenofovir treatment reduces liver inflammation[12], and that in non treated CHB patients, hypercytolytic activity of liver NK cells correlated with liver inflamation[6]. Our findings thus suggest that tenofovir-induced reduction of inflammation is not paralleled by altered intrahepatic NK cell numbers. This is also relevant since it was shown that the frequency of intrahepatic NK cells is lower in untreated CHB patients as compared to healthy individuals[6]. It should be noted that our cohort consisted predominantly of HBeAg-negative patients, whereas Zhang et al examined HBeAg-positive CHB patients, and that the assessment of the relative number of NK cells in the liver does not provide information on the absolute intrahepatic NK cell numbers[6]. For ethical reasons, no histological evaluation could be performed by collecting multiple diagnostic core biopsies, and therefore we cannot determine the degree of infiltration of other leukocytes, and whether the results are biased by zonal distribution of inflammation (portal versus lobular) and the grade of fibrosis. It would be interesting to examine this issue in future studies since altered migration or retention in the liver

likely affects the immunopathological processes that may take place.
In the present study, frequencies of cells positive for CD69 and CD25 were overall constant upon viral reduction. However, baseline viral load, notably variable in HBeAg-negative CHB, play a yet further to be determined role in NK cell activation. Although we observed no significant differences, we can not exclude that also variations in ALT levels contribute to the differences in activation profile. Our observations suggest that liver NK cells under low viral load conditions have a different activation profile than liver NK cells under high viral load conditions. Intrahepatic NK cell activation might thus be involved in tenofovir-induced reduction of liver inflammation, but only at baseline low viral load conditions.
We observed no effect of successful tenofovir treatment on NKG2A and NKG2D expression and IFN-γ production by NK cells (data not shown), which is in contrast to our previously reported findings on blood NK cells following entecavir therapy[4]. The choice of NUC used for viral load reduction, as well as different patient characteristics may explain this discrepancy, and demonstrate the need for further studies to delineate the role of NKG2A on NK cell function during anti-CHB drug therapy. Moreover, since only low numbers of cells can be isolated from a fine needle aspiration biopsy, we choose to first determine intrahepatic NK cell numbers and activation status during therapy of CHB patients, as this elaborate work has never been done. Future studies aimed at understanding the effect of treatment on the intrahepatic NK cell compartment should focuss on functional asays.
In conclusion, we provide a unique insight in the intrahepatic NK cell compartment in CHB patients during tenofovir treatment. Our study demonstrate modest changes in the frequency and activation of liver NK cells after succesful antiviral therapy. Our current findings therefore do not provide evidence that the intrahepatic NK cell response contributes to tenofovir-induced clearance of viral DNA from the liver, and can not be regarded as an outcome of tenofovir therapy.

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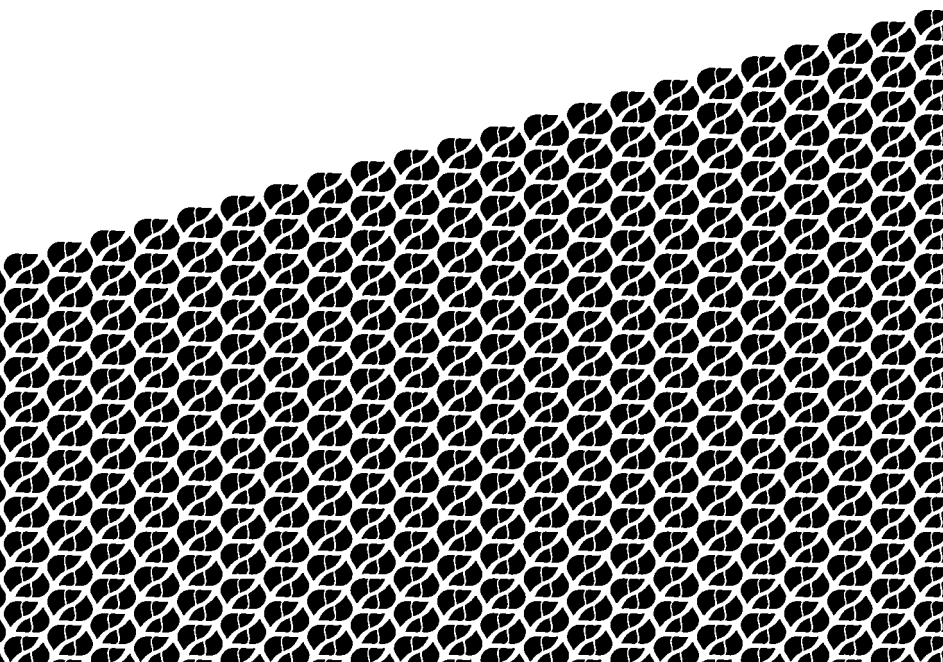

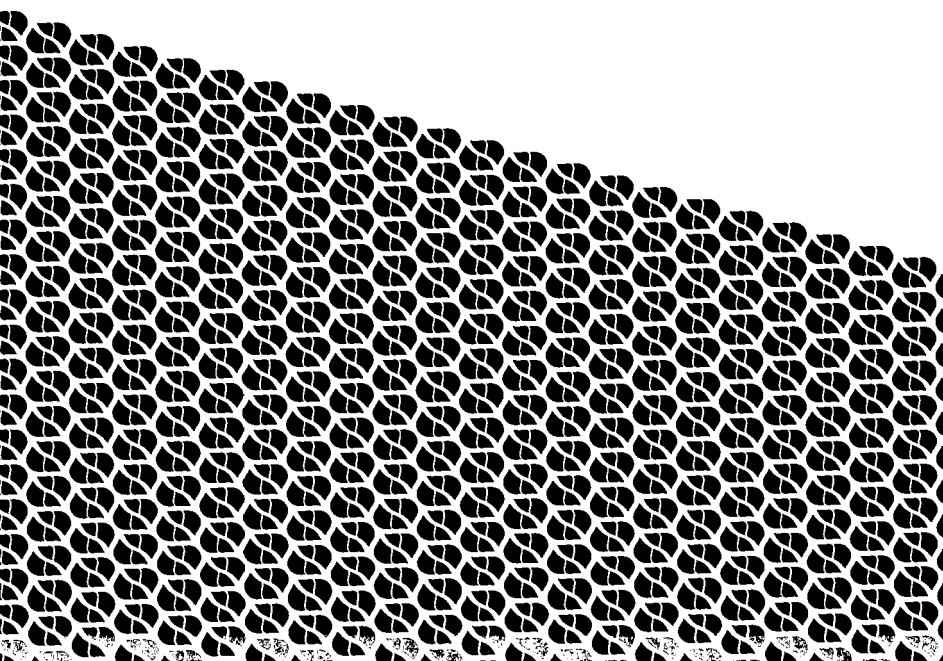

CHAPTER 7

GENERAL: DISCUSSION AND FUTURE PERSPECTIVES

Until recently, research in chronic hepatitis B (CHB) has been focussed on the impaired adaptive immune response leading to viral persistence[1-2]. Induction of adaptive immunity not only depends on direct antigen recognition by the antigen receptors but also relies on essential signals that are delivered by the innate immune system[3]. Animal studies have showed an impaired, weakened and altered innate immune response against HBV[4]. As lymphocytes of the innate immune system, NK cells employ three main strategies to kill virally infected cells: the production of cytokines (notably IFN-γ), the secretion of cytolytic granules and the use of death receptor-mediated cytolysis[5]. NK cells also mediate regulation of the adaptive immune system. First, IFN-γ not only exerts direct effects in making host cells less hospitable to the virus/less susceptible to virus infection but also favours the development of cytotoxic T cells and CD4+T helper type 1 cells[6]. Second, reciprocal interactions with dendritic cells (DC), the other key player in innate immunity, result in a potent activating crosstalk that regulates both the quality and the intensity of the innate immune response as well as the adaptive response.

In this thesis, we demonstrated that total numbers of blood and liver NK cells of CHB patients do not differ compared to controls and remained stable upon antiviral nucleot(s)ide analogue therapy with entecavir or tenofovir (chapter 2, 4, 5 and 6). Circulating NK cells were less activated compared to controls (chapter 2). Compared to peripheral blood, more activated NK cells are present in the liver, but the capacity to get further activated by stimulation is limited compared to their circulating counterparts (chapter 5). The direct cytotoxic properties of circulating NK cells of CHB patients were not different from controls. However, the capacity to produce cytokines upon stimulation was hampered in CHB (chapter 2) and was less in liver NK cells compared to circulating NK cells (chapter 5). The capacity to produce IFN-γ upon stimulation during therapy was significantly improved in circulating NK cells (chapter 2 and 4). This was correlated with the increased ability to become further activated upon stimulation (chapter 2). In contrast to the peripheral blood NK cells, the activation status of liver NK cells of CHB patients is not augmented upon therapy (chapter 6). Also the activation of NK cells by DC *ex vivo* was shown to be impaired in CHB (chapter 3 and 4). This DC-NK cell crosstalk could be enhanced upon antiviral therapy (chapter 4).

POSSIBLE EXPLANATIONS ON IMPAIRED NK CELL ACTIVATION AND FUNCTION IN CHB

Based on the findings in this thesis, we can argue possible explanations on the impaired NK cell activation and function in CHB. These include: a direct effect of the virus on NK cells, the presence of an immunosuppressive milieu and host-limited factors.

1. DIRECT EFFECT OF THE VIRUS ON NK CELLS. Viruses employ immune evasion strategies in order to survive within the host. Innate immune cells may represent a primary target[7]. For instance, HBsAg hampers direct antiviral cytokine production and antigen presentation by different subsets of DC[8-10] and controversial findings report on binding of recombinant HCV envelope protein E2 to its receptor CD81 and directly inhibiting NK cell function[11]. Some findings in this thesis support the hypothesis that HBV inhibits innate immunity: liver NK cell activation is correlated with HBsAg levels (chapter 5), HBV significantly inhibited pDC-induced cytokine production by peripheral blood NK cells *in vitro* (chapter 3) and mDC-induced NK cell function *ex vivo* is ameliorated upon antiviral therapy (chapter 2 and 4). However, we also showed that NK cells exert normal direct cytotoxicity (chapter 2). This would suggest that the direct effect of the virus on NK cells is exerted on a very specific function. However no evidence exist on the presence of HBV in or on NK cells. So far, we were not able to show the presence of viral DNA in NK cells isolated from the peripheral blood of chronic HBV patients (data not shown). Furthermore, HBV was not able to interfere with cytokine induced NK cell function *in vitro* (chapter 3), indicating that HBV does not exert direct inhibitory effects on NK cells. Interestingly, HBV exerted significant suppressive effects on NK cell function via interaction with pDC, resulting in NK cells with normal cytotoxic capacities, but hampered IFN-γ production thereby completely overlapping the functional dichotomy as observed in CHB patients. Thus, these data suggest hampered DC-NK cell crosstalk rather than a direct effect of the virus on NK cells. Next to infectious viral particles, also viral proteins including HBsAg and HBeAg

are present in patients' circulation. HBeAg does not seem to play an important role in liver NK cell activation and function since no differences could be observed in HBeAg positive and HBeAg negative CHB patients (chapter 5). The positive effect of antiviral therapy on DC and NK cell function may be explained through reduction of HBsAg levels, but enhanced NK cell activation was not observed in liver NK cells upon antiviral therapy (chapter 6). In addition, stimulation of peripheral blood NK cells in the presence of recombinant HBsAg *in vitro* did not affect activation and IFN-γ production (chapter 5). Therefore, the contribution of a direct effect of the virus to the impairment of NK cell function seems at most, very limited.

2. IMMUNOSUPPRESSIVE MILIEU. NK cell activity is strongly dependent on receptors, cytokines and other cells such as DC. In CHB, the key to viral survival may lie in skewing the balance by which NK cells are regulated towards immunosuppression. Circulating NK cells show a hampered response on stimulation with IL-12/IL-18 and PMA/ionomycin *ex vivo* (chapter 2 and 4) which could be resulting from constant inhibition *in vivo*. Indeed, IL-10 and TGF-β, known inhibitors of NK cell function, have been observed in elevated levels in the circulation of CHB patients[12, 15], but also other factors could play an immune regulatory role. In this thesis, more insight in this issue has been gained.

A) THE INHIBITORY RECEPTOR NKG2A: in order to activate NK cells, it is crucial to overcome the dominant inhibitory signal present in resting NK cells. This may be achieved through receptor crosslinking of activating receptors, but also by downregulation of inhibitory receptors. In our studies, the upregulation of the expression of the inhibitory receptor NKG2A on circulating NK cells was observed in CHB (chapter 2). HLA-E, its natural ligand, was also present and expressed on mDC (chapter 4) and liver tissue (data not shown). However, NKG2A is recycled by trafficking between cytoplasm and cell surface independently from HLA-E expression to ensure continuous presence on the surface ready for inhibition[14]. Successful antiviral treatment is paralleled by

downregulation of NKG2A expression (chapter 2). It is tempting to speculate that enhanced inhibitory signalling through NKG2A *in vivo* explains impaired NK cell activation and function as was also suggested in HCV infection[15] However, chapter 6 shows that NKG2A expression on liver NK cells is not different after viral load reduction suggesting NKG2A is not directly involved. Finally, the downstream tyrosine-based motif of NKG2A delivers inhibitory signals[16-17], suggesting that overcoming this signal results in the ability to become activated rather than increased cytokine production. We thus show that the role of NKG2A is very limited but to which extend NKG2A receptor expression is still relevant in CHB, remains to be further elucidated.

B) INTERACTION WITH DC: bidirectional activation between NK cells and DC is highly relevant in regulation of the immune response[18]. Impaired activation by activated DC through cytokines and/or cell-cell interaction may underlie impaired NK cell activation and function. Indeed in our studies, NK cell activation and/or cytokine-production upon co-culture with mDC and pDC was less in CHB or in the presence of HepG2.2.15 derived HBV compared to controls (chapter 3 and 4). The cytokine environment mediated by DC may contribute to impaired NK cell activation. mDC derived from CHB patients were less capable to produce IL-12, IL-18 and IL-6 upon stimulation (chapter 4), suggesting this could result in impaired NK cell activation by DC. A second indication that DC and not NK cells are impaired was observed when isolated NK cells from CHB patients were co-cultured with isolated pDC from healthy controls, these NK cells could produce sufficient amounts of IFN-γ (chapter 3). Upon antiviral therapy, the production of cytokines by mDC is ameliorated and DC mediated NK cell activation is enhanced, even of NK cells isolated prior to treatment (chapter 4). This suggest that especially DC function improves upon antiviral therapy, which also has previously been demonstrated[19-20], and underlies at least part of the improved NK cell function.

Next to DC-derived cytokines, also the expression of surface-bound ligands and receptors play an important role in the regulation of

DC-induced NK cell function. However, several of the known NK cell ligands, such as the expression of HLA-ABC , HLA-E and MIC-A on mDC was not altered compared to controls (chapter 4). Another important receptor-ligand pair involved in DC/NK cell interaction is CD244-2B4[19]. The expression of both the ligand and the receptor on blood NK cells, but not on mDC, of CHB patients is increased compared to controls (chapter 3), but the exact relevance of this finding needs to be further elucidated as CD244/2B4 crosslinking results in activation and may play a more distinctive role in crosstalk to T cells compared to DC[21].
In conclusion, it seems more likely that the impaired NK cell function observed in CHB patients is not due to a direct effect of the virus on NK cells, but rather an indirect effect of virus-induced alterations in DC and/or other cells and other immune regulatory networks present as a consequence of a chronic inflammatory state.

3. HOST FACTORS. Impaired NK cell activation and function may be limited to the host and thus due to an intrinsic defect of NK cells. An intrinsic impairment in NK cell function may have significant consequences for antiviral immunity and may hence partially explain the viral persistence. And although patients chronically infected with HBV are not immune compromised and seem to respond adequately to other viral invaders, the survival of HBV by specific targeting of the liver, which is full of NK cells compared to other organs, may benefit from a reduced NK cell function. It is therefore interesting to note, that the capacity to exert direct cytotoxicity via perforin/granzyme mediated pathways was normal as compared to controls, since this pathway is thought to be less relevant in the combat with HBV-infected cells as hepatocytes are not sensitive to direct cytotoxic killing[22]. Nevertheless, it is highly unlikely that the observed impairment of NK cells in CHB patients is intrinsic, since antiviral treatment could significantly improve the function of NK cells (chapter 2 and 4). Therefore, more than an intrinsic defect, impaired NK cell function seems to be secondary to an immunosuppressive milieu, as has been proposed in the previous paragraph.

WHAT DOES IT MEAN IN CLINICAL PRACTICE?

Whether impaired NK cell activation and function are responsible for the induction and/or maintenance of viral persistence and thus CHB is still not known. Isolation of (liver) NK cells prior to and during acute infection with HBV would enable more definite conclusions. Unfortunately, research on immune cells during acute HBV infection in a controlled fashion is only feasible in primate research and in this, even rarely leads to chronicity[25]. Impairment of NK cell activation and function, as is shown in this thesis, has no direct clinical consequences for the CHB patient. In contrast to the autosomal-recessive Chediak-Higashi syndrome in which affected individuals have no or very low NK cell activity[24], CHB patients do not suffer from multiple viral insults.

Nevertheless, antiviral treatment significantly improves NK cell function (chapter 2 and 4) and modulating NK cells may have implications for the current management of CHB. Understanding how the immune system is involved in disease will eventually contribute to the development of novel treatments. Numerous examples such as anti-TNFα therapy in rheumatoid arthritis and inflammatory bowel disease, have shown that patients benefit from immunological research. The use of IFN-α also proved a major breakthrough in the treatment of chronic viral hepatitis[25]. The next challenge for clinicians treating CHB is how to individualize treatment in order to optimize its efficacy and costs. Within the two treatment options currently available, eg. antivirals and immunomodulatory IFN-α, we have been strict in using exclusively one or the other option[26], since combining these two options has only been minimally successful[27]. Unfortunately, these treatment options are not very effective in inducing HBV-specific immune control and hence most patients require life-long treatment and remain at risk for the existence of resistant viral mutants, liver cirrhosis and liver cancer. Therefore, a new effective therapeutic strategy aiming at finite treatment with life-long immune control over HBV is required. At present, we do not fully understand the function of NK cells in CHB due to lack of data on intrahepatic NK cells, the heterogeneity of the disease, etc. Nevertheless, the data presented in the current thesis support a novel concept of

treatment, i.e. first reducing viral load, HBsAg and/or inflammation with antivirals enabling the immune system including NK cells to improve their function and sensitivity for immune activation, followed by immune activation with PEG-IFN-α therapy or another type of immune stimulation. Currently, a phase IV multicentre randomised clinical trial has started investigating this strategy (www.clinicaltrials.gov/NCT00877760).

FUTURE DIRECTIONS

A very important issue that must be accounted for when translating (future) NK cell research into clinical practice is the multiphasic character of CHB. The differentiation into 4 phases based on HBeAg seroconversion and hepatic necro-inflammation[25] needs to be put in perspective since it simply has no well-established patho-immunological basis. Seroconversion of HBeAg only occurs in wild-type virus and not in the prevalent pre-core mutants. In that same respect, HBV has at least 6 genotypes, each with different geographical preference. From HCV infection, it is known that different genotypes can elicit different immune responses[27] and there are some indications this could also account for HBV[29]. Furthermore, the therapeutic response rates to PEG-IFNα is better in terms of HBsAg seroconversion for HBeAg positive disease. This suggests that underlying immune mechanisms may be completely different from HBeAg positive and HBeAg negative disease. Until now, most research on the immune response against HBV has not integrated these issues hampering full implementation for its findings into daily clinical practise. When performing future research, one should better define patient populations with regard to genotype, HBeAg status, ALT level, serum HBsAg levels and preferably duration of infection in order to fully understand its findings.

A second recommendation can be made concerning what compartment to study. It would be most informative to study the intrahepatic compartment, given that after 1-2 weeks of infection,

HBV covalently closed circular (ccc) DNA and HBV replicative DNA intermediates can be traced in the liver[51]. NK cells constitute one-third of the intrahepatic lymphocyte population and also in liver, viral DNA is controlled by non-cytolytic mechanisms[4]. Whether this is mediated through resident liver NK cells or chemokine-mediated recruitment of activated circulating NK cells is unknown. For obvious reasons, most research has been performed on peripheral blood but we should increase our efforts to perform simultaneous intrahepatic studies such as in chapter 5 and 6. An in-depth analysis of NK cells in healthy livers compared to diseased livers will be enormous helpful to better understand the intrahepatic immune response in health and disease, but is due to obvious ethical restrains hardly possible.
In conclusion, as there is now a substantial body of information on NK cells in CHB, we are challenged to make progress to how they may be manipulated in order to generate a successful anti-viral immune response. Finally, our understanding of NK cell biology continues to evolve and thus will continue to generate novel ideas related to viral eradication and disease progression.

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NEDERLANDSE SAMENVATTING VOOR LEKEN

Chronische hepatitis B infectie is een chronische leverziekte met wereldwijd meer dan 450 miljoen geinfecteerden. De ziekte wordt veroorzaakt door de chronische aanwezigheid van het hepatitis B virus (HBV) wat zich met name in de lever ophoudt. Een infectie met HBV wordt vaak verkregen door verticale transmissie, wat inhoudt dat moeders het virus rondom de bevalling overdragen op hun kinderen. Ook kan horizontale transmissie (van volwassene op volwassene) optreden door bijvoorbeeld seksueel contact. Het merendeel van de geinfecteerden kan het virus klaren en is dus niet chronisch geinfecteerd. Deze klaring is het gevolg van een goed werkend immuunsysteem. Bij chronische hepatitis B infectie werkt de antivirale immuunrespons niet adequaat, maar het is niet bekend op welke manier en in welke mate. Op dit moment zijn de therapieën voor chronische HBV infectie onvoldoende effectief, gaan gepaard met veel bijwerkingen en moeten levenslang gevolgd worden. Het is dus belangrijk om de gestoorde antivirale immuunrespons te begrijpen om met die kennis effectievere therapieën te ontwikkelen.
De immuunrespons valt uiteen in een vroege (innate) en een late (adaptieve) immuunrespons. De adaptieve immuunrespons tegen HBV bestaat uit het ontstaan van HBV-specifieke afweercellen (T- en B-cellen) die stoffen zoals cytokines en antilichamen tegen HBV-moleculen of HBV-geinfecteerde levercellen produceren. Deze respons is tot dusver veelal onderwerp van onderzoek geweest en er is aangetoond dat deze niet goed ontwikkeld is. De innate immuunrespons bestaat uit de snelle herkenning van lichaamsvreemde moleculen zoals HBV door speciale cellen (zoals dendritische cellen en NK cellen) en de directe afweer door middel van cytokines en celdodende enzymen. Daarnaast activeren deze cellen het adaptieve immuunsysteem doordat zij de lichaamsvreemde moleculen als het ware presenteren aan de T- en B-cellen. Ondanks deze schijnbare cruciale rol van dit innate immuunsysteem in de afweerrespons tegen HBV, zijn enkel dendritische cellen (DC) enig onderwerp van onderzoek geweest. Hieruit bleek dat DC van chronisch geinfecteerde HBV patiënten inderdaad minder goed hun functie kunnen uitoefenen. Over "natural killer" of NK cellen is veel

minder bekend, ondanks het feit dat van alle afweercellen in de lever en bloed respectievelijk ongeveer eenderde en eenzesde NK cellen is.

In dit proefschrift is de onderliggende **HYPOTHESE** dat een gestoorde innate immuunrespons, en NK cellen in het bijzonder, bepalend is voor de ineffectieve adaptieve immuunrespons tegen HBV. Dit heeft chronische infectie tot gevolg. Door de aanwezigheid van het virus is het niet mogelijk om bij chronisch HBV patiënten te onderzoeken of de NK cel respons de oorzaak of het gevolg is van infectie. Het **DOEL VAN HET PROEFSCHIFT** is derhalve het verrichten van translationeel onderzoek door middel van het bestuderen van fenotype en functie van NK cellen uit zowel bloed als lever van chronisch HBV patiënten, maar ook voor en na antivirale therapie.
In **HOOFDSTUK 2** worden de resultaten beschreven van een studie over de verschillen in fenotype en functie van bloed NK cellen van chronisch HBV patiënten en gezonde controles. Ook werden de chronisch HBV patiënten behandeld met het antivirale medicijn entecavir om het effect van therapie op NK cellen uit bloed te bestuderen. In **HOOFDSTUK 3** en **4** gaat het onderzoek dieper in op de interactie tussen de cellen van de innate immuunrespons, namelijk de DC en de NK cellen. Een gestoorde interactie zou namelijk ook kunnen bijdragen aan de gestoorde immuunrespons. Hoofdstuk 3 beschrijft de interactie tussen bloed NK cellen en de plasmacytoide DC in de aan- en afwezigheid van HBV *in vitro*. Deze subset van DC is vooral van belang door de massale productie van het antivirale cytokine alfa-interferon. In hoofdstuk 4 wordt de interactie met myeloide DC van chronisch HBV patiënten belicht. Deze interactie wordt ook bestudeerd tijdens antivirale therapie met de medicijnen entecavir en tenofovir.
In **HOOFDSTUK 5** en **HOOFDSTUK 6** worden de resultaten beschreven van twee studies verricht met lever NK cellen. Wij analyseerden het fenotype en functie van NK cellen uit leverbiopten van twee subgroepen van chronisch HBV patiënten. Ook verrichtten wij een uniek onderzoek naar de NK cel respons in de lever tijdens antivirale therapie met tenofovir.
Tenslotte wordt in **HOOFDSTUK 7** een beschouwing gegeven van alle bevindingen uit dit proefschrift in het licht van het huidige en toekomstige onderzoeksveld.

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OVER DE AUTEUR

De auteur van dit proefschrift (Leiden, 1976) voltooide zijn voortgezet onderwijs aan het Bernardinus College in Heerlen. Daarna werd de medische opleiding en het artsexamen *cum laude* afgerond aan de Katholieke Universiteit Nijmegen. Gedurende deze opleiding werd meer dan 1 jaar lang wetenschappelijk onderzoek in het buitenland verricht. De basale principes en labvaardigheden in de neuro-gastroenterologie en darmmucosale immunologie werden onder supervisie van respectievelijk Keith Sharkey (Calgary, Canada), Leo Dieleman en Balfour Sartor (Chapel Hill, USA) verkregen.
De opleiding Gastroenterologie en Hepatologie werd aangevangen in het Sint Radboud Ziekenhuis in Nijmegen onder supervisie van Jan Janssen. Tijdens de vooropleiding Inwendige Geneeskunde (Rijnstate Ziekenhuis, Arnhem) werd mede dankzij Rob van Leusen en Clemens Richter interesse voor hepatologie en in het bijzonder chronische hepatitis B gewekt. Op uitnodiging van Solko Schalm kon deze interesse verder vorm krijgen tijdens een langdurige stage Hepatologie en Levertransplantatie in het Erasmus Medisch Centrum te Rotterdam.
De inmiddels hernoemde opleiding tot MDL arts werd vervolgd bij Chris Mulder in het Vrije Universiteit Medisch Centrum te Amsterdam.
De laatste fase van de MDL opleiding werd in 2006 afgebroken en een 3-jarig promotietraject kon worden aangevangen.
De opleiding MDL met aandachtsgebied hepatologie werd (eindelijk volgens velen) afgerond bij Rob de Man in het Erasmus Medisch Centrum in 2010. Sindsdien is hij als stafarts verbonden aan de afdeling MDL van hetzelfde ziekenhuis.
Hij is woonachtig in Breda met zijn echtgenote en 2 kinderen.