Regulatory T Cells in Chronic Hepatitis B Virus Infection

Regulatoire T cellen tijdens een chronische Hepatitis B virus infectie

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Introduction

1

Hepatitis B virus infection

Hepatitis B virus (HBV) belongs to the family of hepadnaviruses. Hepatitis B virions are double shelled particles with an outer lipoprotein envelope. HBV is a small DNA virus, containing only 3200 bases in its genome. The genome consists partly of double-stranded circular DNA. One strand known as the minus strand is almost a complete circle and contains overlapping genes that encode both the structural proteins (HBV surface antigen (HBsAg), pre-S and HBV core antigen (HBcAg)) and the replicative proteins (polymerase and X) (1,2). Hepatitis B e antigen HBeAg) is secreted from virus-infected cells (3). The virus is depicted schematically in figure 1.

After entry into the hepatocyte, the virus core is transported to the nucleus without processing. The circular DNA is then converted into a covalently closed circular DNA, which serves as a template for host RNA polymerase II. This enzyme generates a series of genomic and subgenomic transcripts. All viral RNA is transported to the cytoplasm for protein synthesis. In the cytosol the nucleocapsids are assembled. During this process a single molecule of genomic RNA is incorporated into the assembled viral core to serve as a template for DNA synthesis. After the viral DNA has been synthesized, the core particles bud into regions of the membrane bearing the viral envelope proteins (1,2,4).

Infection with HBV in adults usually results in a self-limiting, acute hepatitis, which confers protective immunity and causes no further disease. In 10% of infected adults HBV leads to a chronic infection. Chronic HBV infection is an important risk factor for the development of liver cirrhosis and hepatocellular carcinoma. Worldwide 400 million people suffer from a chronic HBV infection and approximately 1 million people die annually from HBV-related disease (5,6).

Patients with a chronic HBV infection can have normal liver enzymes, no symptoms and normal or near to normal liver histology. These patients, often called a-symptomatic carriers, seem to be tolerant to the virus, but when there is loss of tolerance with emergence of reactive T cell clones, hepatic inflammation and T-cell mediated liver damage may ensue (7). Since HBV is a non-cytopathic virus, liver injury is mainly mediated by the host immune response (8).

HBV can evade the antiviral immune response by mutational escape, however mutational escape is much more common in HCV infections (9). HBeAg is involved in

the evasion of the immune response during a chronic HBV infection. It can modulate the anti-viral T cell response and can therefore contribute to viral persistence (10, 11).

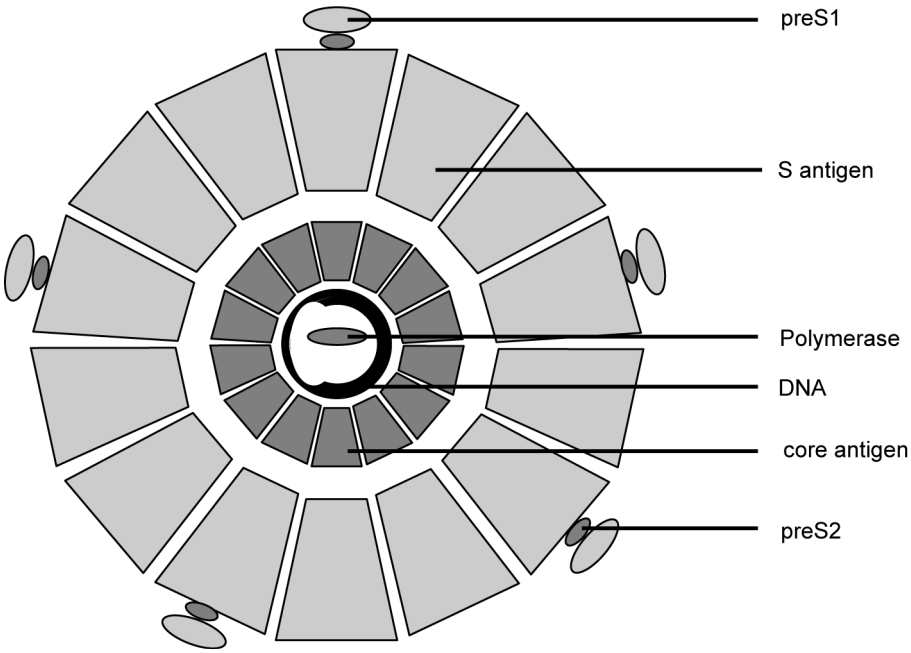

Figure 1. Schematic picture of an HBV-particle, also known as the Dane particle. The outer surface of the virus consists of three types of HBV surface antigen (HBsAg). The viral DNA and the polymerase enzyme reside inside the inner core of the virus.

Treatment of a chronic HBV infection

The goal of treatment for chronic HBV patients is disease remission as indicated by HBV DNA suppression and reduction in liver necroinflammation. The current therapeutic options for patients with a chronic HBV infection include treatment with (pegylated) interferon alpha (IFN-α), lamivudine, adefovir dipivoxil, entecavir and telbivudine. Interferon alpha IFN-α is an immunomodulator while the other drugs are nucleos(t)ide analogs, which primarily act through direct inhibition of viral replication (12). These inhibitors of viral replication, achieve profound reductions in viral load, but usually do not achieve seroconversion for HBeAg (13,14). Treatment with (pegylated) IFN-α is currently the most effective in achieving seroconversion for

HBeAg and a sustained reduction in the peripheral blood HBVDNA levels. However, up till now only 36% of the patients respond to treatment with (pegylated) IFN-α. The exact mechanism of response is unknown thus far (15).

The immunotolerant environment of the liver

The liver appears to be an immune-privileged organ that favors the induction of peripheral tolerance rather than induction of immunity. Hepatocytes and hepatocyte derived cell lines can stimulate HBV-specific CD8+ T cells to produce IFN-γ, but their stimulatory capacity is much lower compared to cell lines obtained from other tissues. The stimulatory capacity of the hepatocytes is dependent of the level of viral antigen present (16). Besides the parenchymal hepatocytes, the liver contains other cell populations: sinusoidal endothelial cells, Kupffer cells, perisinusoidal (stellate) cells, biliary epithelial cells, natural killer (NK) cells, natural killer T (NKT) cells, dendritic cells and lymphocytes. The liver sinusoidal endothelial cells (LSEC) constitute the wall of the liver sinusoids. The LSEC can take up antigen and present this to T cells (17,18). Antigen presentation to CD8+ T cells by LSEC does result rather in tolerance than effector functions. CD8+ T cells co-cultured with LSEC exhibit low IL-2 and IFN-γ production (18). Kupffer cells and LSEC continuously express the immunoregulatory cytokines IL-10 and TGF-β (19). Liver myeloid dendritic cells (mDC) have an increased production of the immunoregulatory cytokine IL-10 as compared to mDC from peripheral blood (20).

The T cell response during a chronic HBV infection

T helper 1 type cytokines such as IFN-γ and Interleukin (IL)-2 are involved in cell-mediated immunity and play a crucial role in the protection against intracellular pathogens, including HBV (21). In patients with an acute self-limiting HBV infection, a multispecific CD4+ and CD8+ T cell response with a type 1 cytokine profile, is important for control of the infection (22). Many different T cell epitopes have been defined in the various HBV proteins. In acutely infected patients, the CD4+ T cell response is principally focused on nucleocapsid antigens of HBV, while the CD8+

T cell response targets epitopes within all viral proteins (8). HBV-specific CD8+ T-cell responses have predominantly been assessed in HLA-A2-positive individuals by using tetramer technology. In almost all HLA-A2 positive individuals who were able to clear HBV, CD8+ T-cell responses specific for the core epitope (c18–27) were detected (8).

The multispecific T cell responses are maintained for decades after clinical recovery (23). In contrast, patients with a chronic HBV infection lack such a vigorous multispecific response. These patients have a weak or undetectable virus-specific CD4+ and CD8+ T cell response (22).

Some chronic HBV patients without evidence of liver damage and low viral load (immune control patients) do have detectable HBV core specific CD8+ T cells in their peripheral blood and liver. On the other hand, in patients with a high viral load and a high degree of liver inflammation, circulating HBV core specific CD8+ T cells are undetectable or present in very low levels. Usually these patients have the same absolute number of HBV specific CD8+ T cells in the liver as immune control patients, but these specific T cells are diluted by a much greater infiltration of non-specific T cells. These non-HBV specific T cells are thought to be responsible for the lysis of hepatocytes (24,25).

Noncytolytic immune functions play an important role in controlling an HBV infection (26). IFN-γ produced by HBV-specific activated PBMC can inhibit viral replication without killing the infected hepatocytes (27). In mice models has been shown that IFN-γ production by NKT cells also plays an important role in the inhibition of the viral replication (28,29).

The role of dendritic cells in chronic HBV

In blood, two important dendritic cell (DC) subtypes have been described, i.e. the mDC and the plasmacytoid DC (pDC) (30,31). MDC are potent antigen presenting cells and thus play an important role in the induction of specific T-cell responses (32). MDC are from myeloid origin and characterized by the presence of markers such as CD1c (blood dendritic cell antigen-1 (BDCA1)) and CD11c and the absence of surface expression of lineage markers. In response to bacterial compounds or CD40 ligand, mDC can produce large amounts of IL-12 (31). PDC,

on the other hand, are from lymphoid origin and are characterized by the presence of BDCA2, BDCA4 and CD123 (IL-3 receptor α-chain). PDC can produce large amounts of type I interferon (IFN), IFN-α and IFN-β, upon exposure to viruses as well as bacterial components such as CpG oligonucleotides (33-35). In case of a viral infection, both mDC and pDC are likely to be exposed to the invading virus and play a critical role in the control of the infection. MDC and pDC of chronic HBV patients are functionally impaired compared to mDC and pDC from healthy controls (36-38). Purified mDC were less capable of full maturation after stimulation as was determined by a reduced upregulation of the co-stimulatory molecules CD80 and CD86, they have a reduced T cell stimulatory capacity, and they exhibit a reduced capacity to produce the immunostimulatory cytokine tumor necrosis factor (TNF)-α (36). TNF-α has a direct anti-viral effect and is essential for the proliferation of HBV-specific cytotoxic T cells (39-41). In addition, isolated pDC from chronic HBV patients produced less IFN-α (36). Therapy induced viral load reduction with adefovir has a favorable effect on the number and functionality of mDC of patients with chronic HBV. For lamivudine induced viral load reduction a favorable effect on pDC was recently shown. (38,42).

Regulatory T cells

Several different populations of T cells with a regulatory phenotype have been described in literature, such as those that secrete IL-10 (TR-1 cells) or TGF-β (T helper3 cells) (43). The best defined population of regulatory T cells are the CD4+CD25+ regulatory T cells (Treg). Treg are of major importance to maintain peripheral immune tolerance (44-46). The development and the severity of an autoimmune disorder is often associated with a decreased number of circulating Treg (47-49). On the other hand, increased numbers of Treg can cause immune hyporesponsiveness, which contributes to the chronicity of pathogenic infections and to the immunotolerance to tumors (50-54). Treg are therefore considered as a potential agent for immunomodulatory therapy and there is much interest in their ex vivo expansion (55).

Treg can be distinguished from CD4+ effector T cells by their continuous expression of the activation marker CD25 and the expression of the Treg specific

transcription factor *FoxP3. FoxP3* is a key regulatory gene for the development of Treg. Transfection of *FoxP3* converts CD4+ effector T cells to Treg (56,57). Mutations in the *FoxP3* gene have been shown to result in a loss of Treg function in mice models (scurfy mutation) as well as in patients with the immunodisregulation, polyendocrinopathy and enteropathy, X linked syndrome (IPEX) (58). In addition to CD25 and FoxP3, several other markers such as cytotoxic T-lymphocyte antigen 4 (CTLA-4), Glucocorticoid-induced-tumor-necrosis-factor-related-protein (GITR), HLA-DR, OX40 and Lymphocyte-activation gene 3 have been associated with Treg. (43). Treg can also express membrane bound TGF-β (59). CD127 expression is inversely correlated with FoxP3 expression and suppressive capacity of Treg (60).

Treg are capable of inhibiting the effector functions of $CD4^+$, $CD8^+$, NK cells and NKT cells (45,61-65). Treg can also indirectly inhibit the antibody production by B cells via inhibition of CD4+ T cells (66). Besides suppressing the immune response, Treg can also have an activating effect on the immune system. In a recent study by Xu *et al* is shown that Treg can induce the development of the T helper 17 cells in the presence of IL-6 (67).

Although Treg were originally thought to be of thymic origin there is also evidence of peripheral Treg generation. Treg are thought to have a high turnover and therefore proliferation of Treg from thymic origin or de novo Treg induction is necessary for the lifelong maintenance of this cell population (43,68). TGF-β is involved in the peripheral induction of Treg. Stimulation of CD4+ T cells in the presence of TGF-β results in the induction of Treg. There is some evidence that besides TGF-β, CTLA-4 is required for this process (69,70). Treg of thymic origin are often referred to as naturally occurring Treg and Treg which are induced in the periphery are often named adaptive or induced Treg. The phenotype of these two different Treg populations as well as their mechanism of suppression is remarkably similar. It is therefore not always possible to make a distinction between the two subtypes (43).

The mechanism of the suppressive activity exerted by Treg on the T cell response remains unclear and there is inconsistency in the literature about the possible involvement of the immunoregulatory cytokines IL-10 and TGF-β. It appears that *in vitro*, suppression by Treg is contact dependent (51,71-73). In mice models the negative regulator of T cell activation CTLA-4 is involved in Treg mediated suppression (74). However in most *in vitro* studies with human Treg neutralization of CTLA-4 with antibodies did not affect Treg mediated suppression (61,75,76).

Because of the central role Treg play in the regulation of the adaptive immune response and their increased presence during several diseases characterized by immuno-hyporesponsiveness, they might also be a mediator of the impaired immune response in chronic HBV.

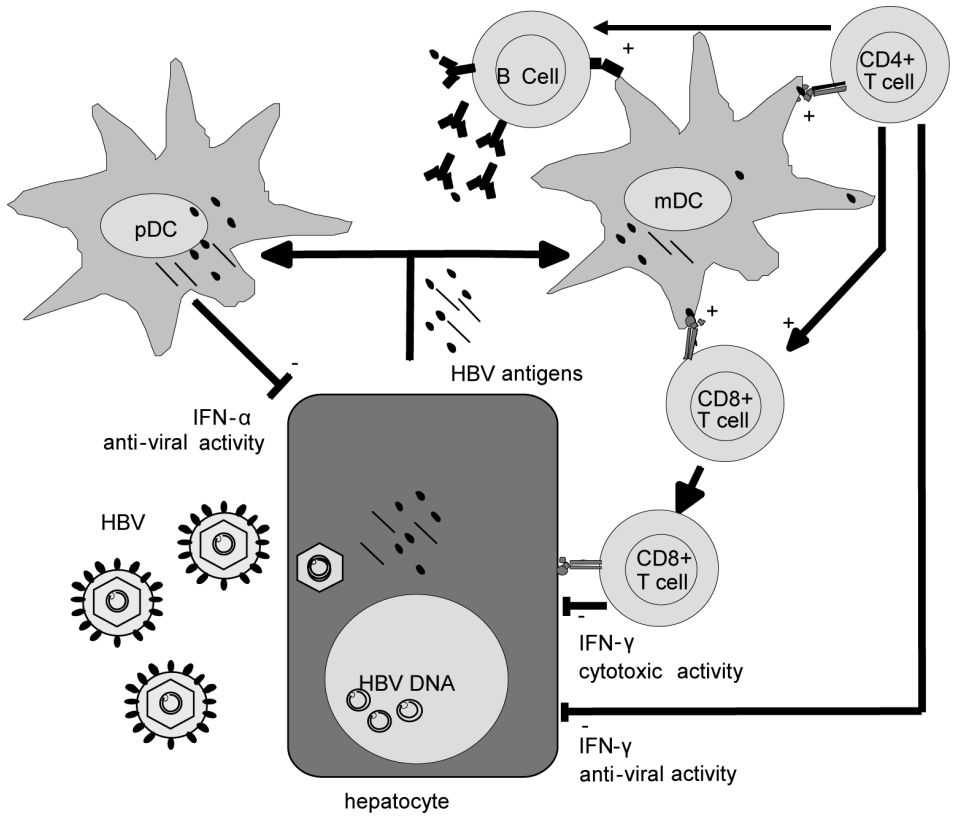

Figure 2. Schematic overview of the different interactions between cells of the adaptive immune system, after a hepatocyte is infected with HBV. An arrow accompanied by a plus symbol indicates a stimulatory signal and an arrow accompanied by a minus symbol indicates an inhibitory signal.

Scope of this thesis

The scope of this thesis is to gain more insight into the possible role of Treg in the impaired immune response during chronic HBV infection. In this thesis we focus on the difference in the proportion of Treg between patients with a chronic

HBV infection, individuals with a resolved HBV infection and healthy controls. We also assessed the capacity of Treg to inhibit the anti-HBV response. Since the liver is the site of infection, we have also studied the proportion of intra hepatic Treg. Furthermore, we have studied the interaction between Treg and DC and the mechanism by which Treg suppress the anti-HBV response *in vitro*. Finally, Treg have been studied during treatment to try to determine whether they might contribute to or inhibit the effectiveness of therapy. Monitoring Treg during anti-viral therapy also provided us with the opportunity to study the effect of the viral load on the proportion of peripheral blood Treg.

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Regulatory T cells contribute to the impaired immune response in patients with a chronic hepatitis B virus infection

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Abstract

Chronic hepatitis B virus (HBV) infection is characterized by a weak immune response to HBV. Regulatory T cells (Treg) can suppress the function of effector T cells and may thus be key players in this impaired immune response. Changes in the functionality or number of Treg could explain the decreased antiviral response in chronic HBV patients. To investigate the role of Treg in chronic HBV infection, we compared the proportional frequency and functionality of Treg in peripheral blood of 50 chronic HBV patients, 23 healthy controls and 9 individuals with a resolved HBV infection. A higher percentage of Treg, defined as CD4, CD25, CD45RO and cytotoxic T lymphocyte-associated antigen-4 positive cells, was detected within the population of $CD4^+$ cells in peripheral blood of chronic HBV patients compared to healthy controls and individuals with a resolved HBV infection. Accordingly, chronic HBV patients displayed a higher *FoxP3* messenger RNA level than healthy controls. Depletion of the $CD25^+$ cells from peripheral blood mononuclear cells (PBMC) of chronic HBV patients resulted in an enhanced proliferation after stimulation with HBV core antigen. Reconstitution of these depleted PBMC with $CD4^+CD25^+$ Treg resulted in a dose dependent reduction of both HBV-specific proliferation and interferon-γ production. **In conclusion**, chronic HBV patients harbor an increased percentage of Treg in peripheral blood compared with controls. Treg have an immune suppressive effect on the HBV-specific helper T cells. The presence of HBV-specific Treg could contribute to an inadequate immune response against the virus, leading to chronic infection.

Introduction

Hepatitis B virus (HBV) is a common non-cytopathic DNA virus. Infection with HBV in adults results frequently in a self-limiting, acute hepatitis, which confers protective immunity and causes no further disease. In 10% of infected adults HBV leads to a chronic infection. Chronic HBV-infection is an important risk factor for the development of cirrhosis and hepatocellular carcinoma. Worldwide, 350 million people suffer from chronic HBV infection, and approximately 1 million people die annually from HBV-related liver disease (1,2).

T helper 1 type cytokines such as interferon γ (IFN-γ) and interleukin 2 are involved in cell-mediated immunity and play a crucial role in the protection against intracellular pathogens, including HBV (3). In patients with an acute self-limiting HBV infection, a multispecific $CD4^+$ and $CD8^+$ T cell response with a type 1 cytokine profile is important for control of the infection (4). These multispecific T-cell responses are maintained for decades after clinical recovery (5). In contrast, patients with a chronic HBV infection lack such a vigorous multispecific response. These patient have a weak or undetectable virus-specific T-cell response (4). The precise mechanism responsible for this T-cell hyporesponsiveness or tolerance is still unknown. One scenario that has not been explored in relation to chronic HBV infections is the potential role of host-mediated immunosuppressive mechanisms that might be activated in the face of persistent antigenic exposure.

Peripheral T cells contain an immunoregulatory sub-population which expresses CD4, CD25 (the interleukin 20 receptor α chain) and CD45RO, as well as the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). These regulatory T cells (Treg) are capable of inhibiting the effector functions of $CD4^+$, $CD8^+$, and natural killer T cells (6-9). Treg express the forkhead/ winged helix transcription factor gene (*FoxP3*). Retroviral gene transfer of *FoxP3* converts naïve T cells into $CD4^+CD25^+$ Treg capable of suppressing the proliferative response of other $CD4^+$ T cells (10). This indicates that *FoxP3* is a key regulatory gene for the development of Treg. Several studies have shown that Treg play an important role in maintaining the peripheral immune tolerance (7,11,12). Furthermore, there is increasing evidence that $CD4^+CD25^+$ Treg contribute to the immunological hyporesponsiveness against several pathogens, resulting in chronic infections (13-16). It has been suggested that Treg can be induced through repetitive stimulation of T cells by high concentrations of antigen for extended periods (17).

The aim of this study was to determine whether Treg are involved in the inadequate immune response leading to chronic HBV infection. We hypothesized that patients with a chronic HBV infection have a higher proportion of Treg compared to healthy controls and people who have resolved their HBV infection and that Treg may be responsible for the inability of the patients to clear the infection. For this purpose we investigated the amount, phenotype, and function of Treg in the peripheral blood of chronic HBV patients, healthy controls, and individuals with a resolved HBV infection.

Patients and Methods

Patients and Healthy Controls. Heparinized peripheral blood samples were obtained from 50 chronic HBV patients, 23 healthy controls, and 9 individuals with resolved HBV infections (Table 1). Patients coinfected with HIV, hepatitis A virus, hepatitis C virus (HCV) or hepatitis D Virus and patients with a resolved viral hepatitis (other than HBV) were excluded from this study. Patients and controls who were immunocompromised or pregnant and patients that received antiviral or immunomodulatory HBV treatment during the last 6 months before blood sampling were also excluded. All participants gave informed consent before blood donation.

Table 1. Patient and control characteristics

	Chronic HBV patients n = 50	Controls n = 23	Resolved HBV patients n = 9
Sex	27 male (54%)	14 male (61%)	5 male (56%)
Age (Years)	33 ± 1.7	31 ± 2.0	45 ± 5.1
ALT (units/L)*	58 ± 6.6	n.a.	37 ± 8.7
HBV DNA (geq/ml)*	$2.4 \cdot 10^9 \pm 1.7 \cdot 10^9$	n.a.	Neg
HBeAg positive	24 (48%)	n.a.	0

Abbreviations:ALT, alanine transaminase; n.a., not applicable; neg, negative;
* mean ± SEM

Isolation of the PBMCs and Flow Cytometric Analysis. Peripheral blood mononuclear cells (PBMC) from chronic HBV patients and controls were obtained by ficoll separation (Ficoll-Paque^{TM} plus, Amersham Biosciences, Buckinghamshire, UK). The PBMC were immediately frozen in medium containing 10% DMSO and

stored at –135°C until further use. Flow cytometric analysis was performed on the stored samples using fluorochrome- conjugated antibodies specific for the surface markers CD4, CD45RO and CD25 diluted in PBS/ 0.3% bovine serum albumin. The cells were fixed by incubation with intraprep reagent 1 and permeabilized by incubation with intraprep reagent 2 (Beckman-Coulter, Marseille, France). Anti-CTLA-4 antibody was added during permeabilization. The following antibodies were used: anti-CD4-PerCP-Cy5.5 (SK3) (Pharmingen, San Diego, CA), anti-CD45RO-APC and anti-CD45RO-FITC (UCHL1) (Becton Dickinson, San Jose, CA), anti-CD25-FITC and anti-CD25-APC (2A3) (Becton Dickinson), anti-CTLA-4-PE (BNI3) (Immunotech, Marseille, France), anti-CD62L-FITC (FMC46) (Serotec, Oxford, United Kingdom) and anti-GITR-FITC (110416) (R&D systems, Oxon, United Kingdom). For the CD45RO, CD25, CTLA-4 and GITR antibodies, isotype-matched control antibodies were used to determine the level of background staining. After staining the cells were analyzed using a four-color cytometer (FACScalibur^{TM}, CELLQuest Pro^{TM} software, Beckton Dickinson).

$CD4^+CD25^+$ T Cell isolation. Fresh PBMC were used for the $CD4^+CD25^+$ isolation. $CD4^+$ T cells were isolated from PBMC by negative selection using the untouched $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+CD25^+$ T cells were isolated from $CD4^+$ T cells using anti CD25-microbeads (Miltenyi biotec). The isolations were performed according to manufacturer's instructions. The $CD4^-$ and the $CD4^+CD25^-$ fraction were pooled and were used as CD25 (Treg)-depleted responder cells. Purity of the cell fractions was determined by flow cytometry analysis with antibodies against CD3, CD4 and CD25. The following antibodies were used: anti-CD3-FITC (UCHT1) (Immunotech), anti-CD4-PerCP-Cy5.5 (SK3) (Becton Dickinson) and anti-CD25-PE (M-A251) (Pharmingen). An isotype-matched control antibody was used to determine the level of background staining for the CD25-PE antibody. The $CD4^+CD25^+$ Treg purification method resulted in a Treg fraction containing more than 90% pure $CD4^+CD25^+$ Treg and a Treg-depleted cell fraction containing all other cell types present in PBMC.

Proliferation assay. Freshly isolated PBMC and the pooled CD25-depleted cells were cultured in triplicate in a concentration of 1 X 10^5 cells per well in 100 µl RPMI 1640 (Bio Whittaker, Verviers, Belgium) containing 10% pooled human serum (Dept. of Immunohematology and Bloodbank, Leiden University Medical

Center, Leiden, The Netherlands) and penicillin/streptomycin (Gibco, Paisley, UK). The cells were stimulated with 1 μg/ml HBV core antigen (HBcAg) (a kind gift by M. van Roosmalen, Biomerieux, Boxtel, The Netherlands), 5 μg/ml Phytohemagglutin (Murex, Paris, France), 1.5 limits of flocculation/ml purified tetanus toxin (SVM, Bilthoven, The Netherlands) or not stimulated and cultured for 6 days. Inhibition of the proliferation by the $CD4^+CD25^+$ Treg was tested by adding 10% (11,000 cells), 20% (25,000 cells) or 30% (43,000 cells) Treg to 1 X 10^5 CD25- depleted cells in 100 μl RPMI 1640 containing 10% human serum and penicillin/streptomycin. The cells were stimulated with 1 μg/ml HBcAg and cultured for 6 days. After 5 days of incubation 50 μl of the supernatant was harvested for ELISA and replaced with 50 μl identical medium and the cells were pulsed with 0.25 μCi/well of [^{3}H]thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK). The cells were harvested 16 hours later. Proliferation, for both assays, was determined by liquid scintillation counting of the harvested cells. The IFN-γ production was determined by measuring the IFN-γ concentration in the harvested supernatant using a cytokine ELISA kit from U-CyTech (Utrecht, The Netherlands). This assay was performed according to the manufacturer's instructions.

FoxP3 RNA quantification. RNA was isolated from thawed PBMC samples of a subgroup of HBV patients (n=25) and healthy controls (n=11) using TRIzol® (Invitrogen, Breda, The Netherlands). Residual DNA was removed from the RNA sample with the DNA-FREE RNA kit™ (Zymo, Orange, CA). The amount of RNA was quantified using a Ribogreen quantification kit (Molecular Probes, Leiden, The Netherlands). Copy DNA was produced in a reverse transcriptase reaction with a random primer hexamer (Promega, Leiden, The Netherlands) using 750 ng of the RNA. The *FoxP3* messenger RNA levels were determined in a real-time PCR with the TaqMan (Abi Prism 7700 sequence detector, Applied Biosystems, Foster City, CA), using GAPDH as an internal control. The *FoxP3* PCR primers and probe were obtained from Applied Biosystems assay on demand and the GAPDH primers and probe were obtained from Biosource (Camarillo, CA). The relative copy number *FoxP3* was calculated as recommended by the manufactures instructions. The relative amount of messenger RNA per $CD4^+$ cell was determined using the following formula: (copies messenger RNA/ RNA concentration used in the reverse transcriptase reaction)/ percentage of $CD4^+$ cells.

Statistical analysis. Flow cytometry and *FoxP3* data were analyzed with the Mann Whitney U test. Analysis of the depletion assay data was performed with the Wilcoxon matched pairs signed rank sum test. For these analyses SPSS 11.5 for Windows (SPSS, Chicago, IL) was used. Statistical analysis on the reconstitution experiments was performed by analysis of the logarithmic transformation of the dependent variable with random intercept and random slope using PROC Mixed in SAS version 8.2. (SAS Institute Inc, Cary, NC).

Results

Patients with a chronic HBV infection have an increased proportion of Treg in peripheral blood compared to healthy controls. To determine whether $CD4^+CD25^+$ Treg play a role in the persistence of a chronic HBV infection we first compared the percentage of Treg present in the peripheral blood of chronic HBV patients (n=50), healthy controls (n=23) and individuals with a resolved HBV infection (n=9) (Table 1). The percentage of Treg was determined by flow cytometry. Several different markers, which are known to be expressed on $CD4^+CD25^+$ Treg, were investigated. The expression of CTLA-4, CD45RO, CD62L and GITR on the $CD4^+CD25^+$ cells was compared for PBMC from chronic HBV infected patients, healthy controls and individuals with a resolved HBV infection. The $CD4^+CD25^+$ cells with high expression of CD25 all expressed CD45RO and CTLA-4, however, this was not the case for GITR and CD62L (data not shown). Based upon a previous study, which showed that the $CD4^+CD25^+$ cells with high expression of CD25 are Treg (6), CD45RO and CTLA-4 were used as additional markers in our flow cytometry experiments. Typical dot-plots obtained by flow cytometry for PBMCs from a representative chronic HBV patient are shown in Figure 1. The percentage of Treg was defined as the percentage of cells that stained positive for CD4, CD25, CD45RO and CTLA-4 divided by the percentage of cells that stained positive for CD4. Patients with a chronic HBV infection showed a significantly higher percentage of Treg within their population of $CD4^+$ T cells in peripheral blood compared to healthy controls (5.40% ± 0.54% versus 3.23% ± 0.66% (mean ± SEM), p=0.003) and individuals who had resolved their HBV infection (5.40% ± 0.54% versus 2.96% ± 0.61%, (mean ± SEM), $p<0.035$). There was no significant difference in the

percentage of Treg between healthy controls and individuals with a resolved HBV infection (Figure 2).

Chronic HBV patients had a similar increase in the percentage of $CD4^+CD25^{+-}$ $CD45RO^+CTLA\text{-}4^+$ cells in the total lymphocyte population compared to healthy controls and resolved HBV subjects. In addition, we did not observe a difference between patients and controls for the percentage of $CD4^+CD25^+$ cells within the subset of $CD4^+$ T cells (data not shown).

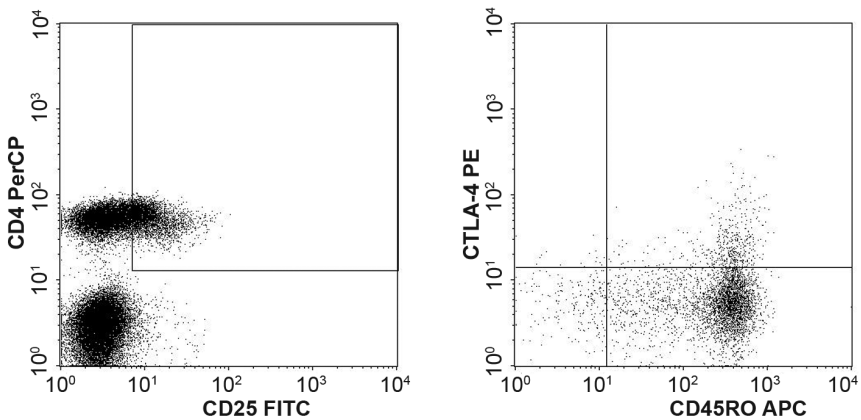

Figure 1. Flow cytometry staining experiment from a representative chronic HBV patient using antibodies against CD4, CD25, CD45RO and CTLA-4. The gates were set using isotype matched control antibodies. The cells depicted in the gate of the left dot plot are $CD4^+CD25^+$ cells and are depicted in the right dot plot. The cells in the upper right quadrant of this dot plot are $CD4^+CD25^+CD45RO^+CTLA\text{-}4^+$ and are considered to be Treg.

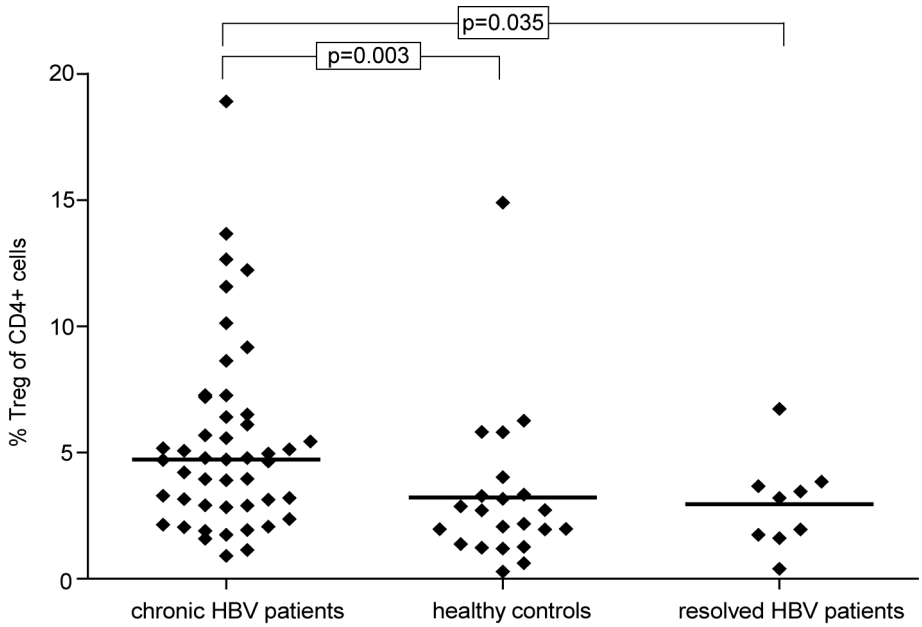

Figure 2. Percentage of Treg (CD4+CD25+CD45RO+CTLA-4+ cells) within the $CD4^+$ cell fraction, determined by FACS staining on PBMC of 50 chronic HBV patients, 23 healthy volunteers and 9 individuals with a resolved HBV infection. The patients have a higher percentage of Treg compared to healthy controls and compared to those who have resolved their HBV infection. The bar represents the median percentage of Treg.

FoxP3 RNA expression is increased in patients with a chronic HBV infection. The relative *FoxP3* messenger RNA (mRNA) levels of the peripheral blood samples from a subgroup of 25 chronic HBV patients and 11 healthy controls was determined by real-time reverse transcriptase PCR. *FoxP3* mRNA levels per $CD4^+$ cells in patients with a chronic HBV infection were significantly higher as compared to healthy controls (204 ± 21.5 versus 96 ± 10.9 (mean ± SEM), p=0.001), respectively (Figure 3).

To assess whether the relative *FoxP3* expression was specific for $CD4^+CD25^+$ Treg, *FoxP3* mRNA levels were determined in isolated $CD4^+CD25^-$ and $CD4^+CD25^+$ cells. $CD4^+CD25^+$ cells contained a 193-fold higher *FoxP3* mRNA level as compared to $CD4^+CD25^-$ cells (2047 ± 721.8 versus 10 ± 4.9 (mean ± SEM)).

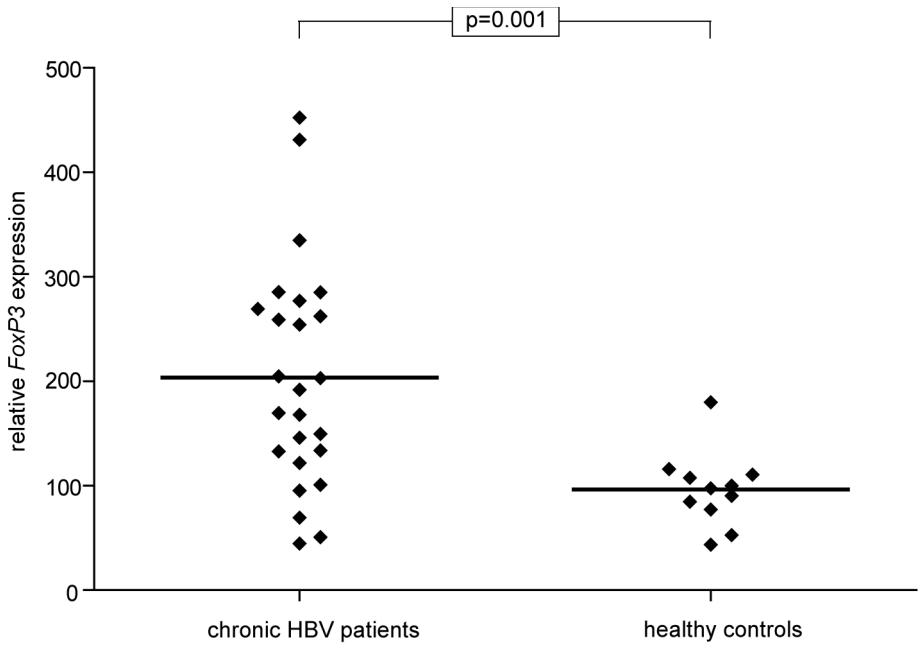

Figure 3. Relative *FoxP3* expression in the PBMC of 25 chronic HBV patients and 11 healthy controls. The relative *FoxP3* mRNA levels are depicted as the number of copies per ng RNA used in the reverse transcriptase reaction. The number of copies is divided by the percentage of $CD4^+$ cells, which was determined in the FACS staining experiments of the same sample. There is a significant higher expression of *FoxP3* mRNA in the patients with a chronic HBV infection compared to healthy controls.

Treg inhibit the HBV core antigen specific proliferation in a dose dependent manner. Next, we determined whether Treg present in peripheral blood of chronic HBV patients can functionally suppress HBV-specific T-cell responses. Depletion of $CD4^+CD25^+$ Treg from PBMC of 17 chronic HBV patients resulted in a significantly stronger proliferation upon stimulation with HBcAg as compared to PBMC (p=0.001) (Figure 4A). For 11 patients the proliferation of Treg-depleted T cells was compared to PBMC after stimulation with purified tetanus toxin. As shown in Figure 4B, there was no increase in proliferation upon stimulation with tetanus toxin after depletion of $CD4^+CD25^+$ cells. PBMC from healthy controls did not proliferate upon stimulation with HBcAg (data not shown). The isolated $CD4^+CD25^+$ T cells were hyporesponsive, as they did not proliferate upon stimulation with the mitogen phytohemagglutin (data not shown).

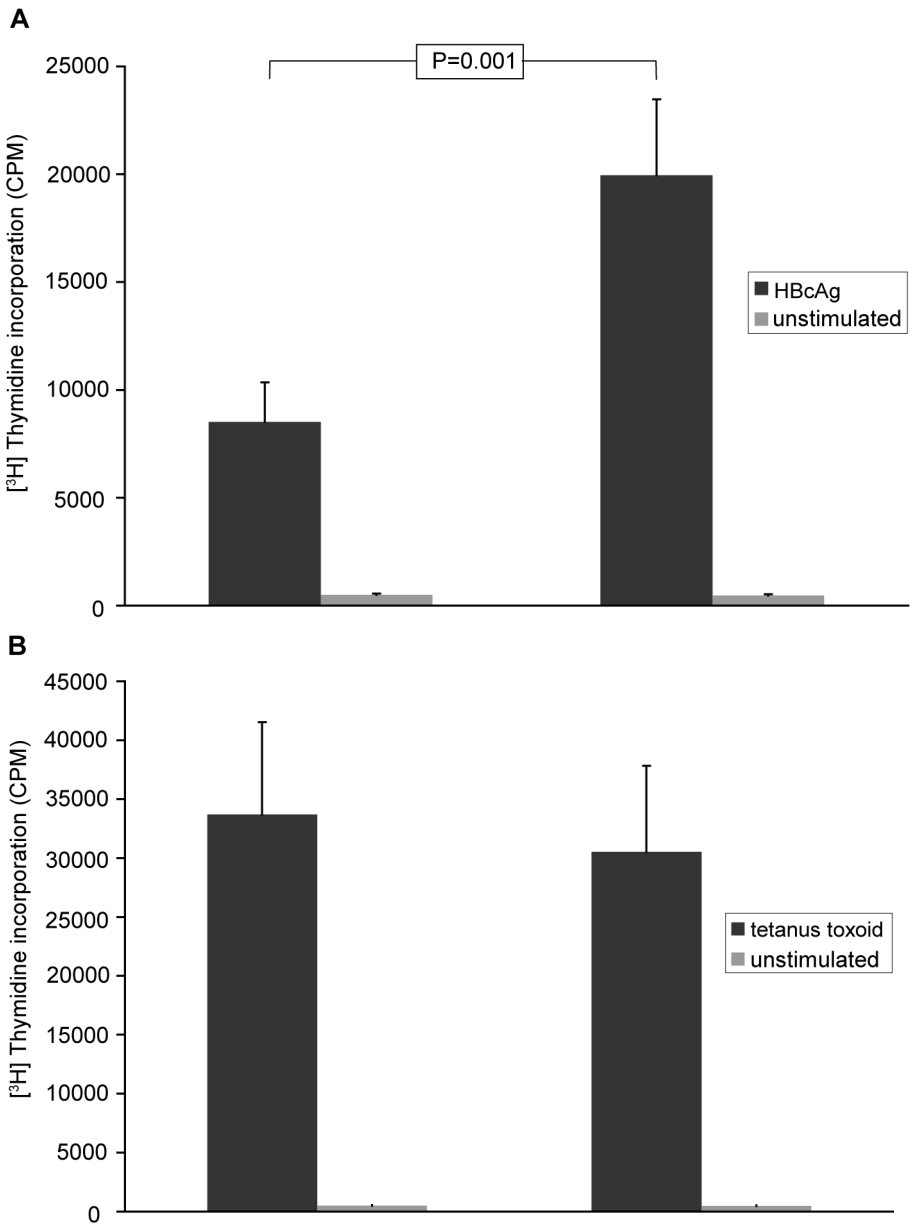

Figure 4. Proliferation of the PBMC and CD25 depleted cells ($CD25^-$). PBMC and CD25 depleted cells were stimulated for six days with HBV core antigen (n=17) or purified tetanus toxin (n=11). The proliferation was determined by the incorporation of [^{3}H]thymidine. The black bar depicts the proliferation upon stimulation with antigen and the gray bar depicts the background proliferation of unstimulated cells. **A)** $CD4^+CD25^+$ Treg depletion resulted in a significant increase of the HBcAg specific proliferation. **B)** $CD4^+CD25^+$ Treg depletion did not result in an increased proliferation upon stimulation with purified tetanus toxin. Data are expressed as mean ± SEM counts per minute (CPM).

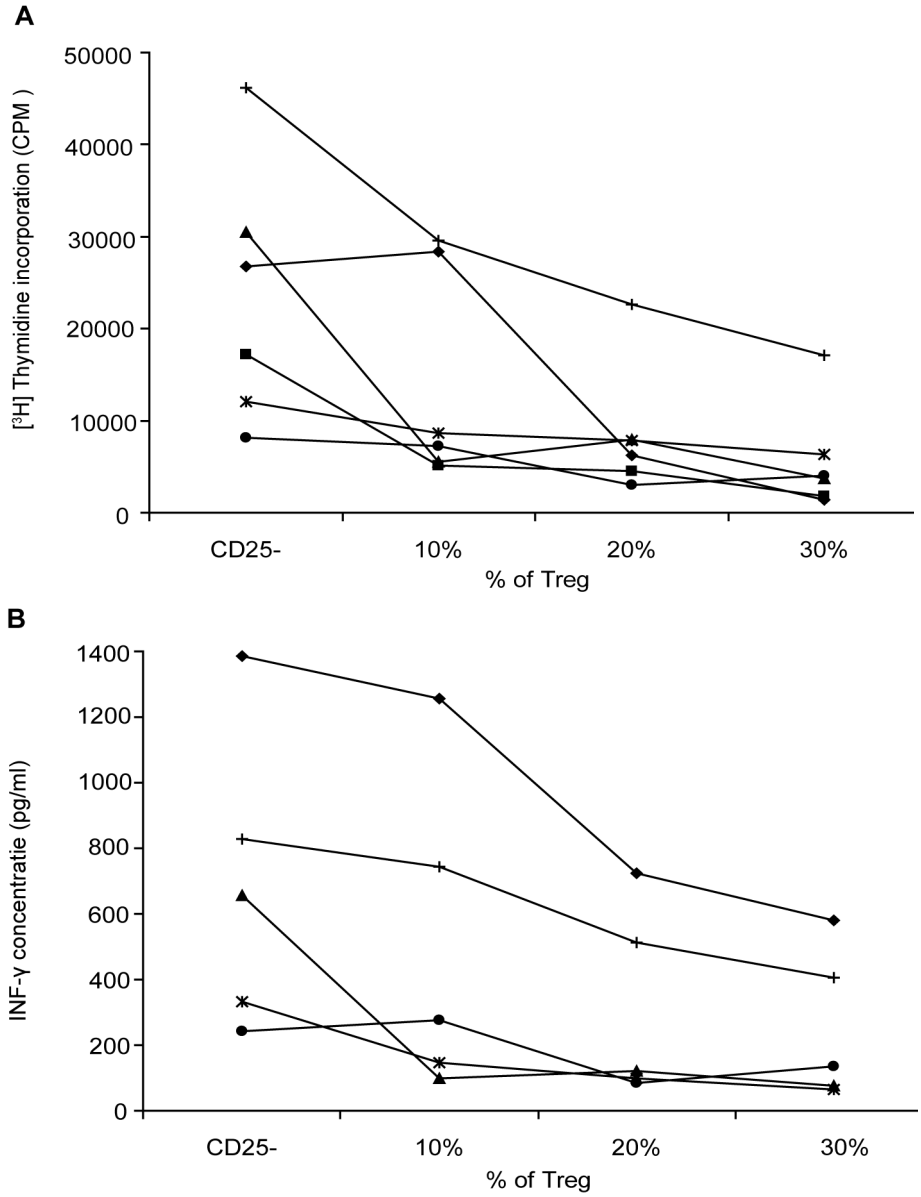

Figure 5. $CD4^+CD25^+$ Treg inhibit the T cell proliferation and IFN-γ in a dose dependent manner. CD25 depleted cells ($CD25^-$) and $CD25^-$ cells reconstituted with 10% (11,000), 20% (25,000) and 30% (43,000) $CD4^+CD25^+$ T cells were stimulated for six days with HBcAg. **A)** The proliferation of the cells was determined by the incorporation of [^{3}H]thymidine (n=6). Lines represent individual patients. Statistical analysis was performed on the logarithmic transformation of the CPM with a random intercept and random slope (estimated population average profile simple linear regression: intercept = 4.28, $p<0.0001$ and mean slope = -0.23, p=0.0091). **B)** The IFN-γ production was determined by ELISA (n=5). Lines represent individual patients (estimated population average profile simple linear regression: intercept = 2.72 $p<0.0001$ and mean slope = -0.018, p=0.0059).

To confirm the suppression of the HBcAg response by Treg, 10% (11,000), 20% (25,000) or 30% (43,000) $CD4^+CD25^+$ T cells were reconstituted to 1 X 10^5 CD25 depleted cells and the proliferation and IFN-γ production was determined. $CD4^+CD25^+$ T cells inhibited the HBcAg-specific proliferation in a dose dependent manner (Figure 5A). The IFN-γ concentration in the pooled supernatant of proliferation assay was determined by ELISA. The IFN-γ production was also inhibited in a dose dependent manner by $CD4^+CD25^+$ T cells (Figure 5B).

Treg in relation to clinical parameters. Among the 50 chronic HBV patients, 24 were HBV e antigen (HBeAg) positive and 26 were HBeAg negative. For analysis of Treg in relation HBeAg we excluded five patients with a pre-core mutant, since these patients are not able to produce HBeAg, irrespective of HBV replication status. HBeAg-positive patients exhibited a higher percentage of Treg in peripheral blood as compared to HBeAg-negative patients (p=0.045) (Figure 6). No correlation was observed between the viral load or hepatic inflammation (serum alanine aminotransferase level) and the percentage of peripheral blood Treg.

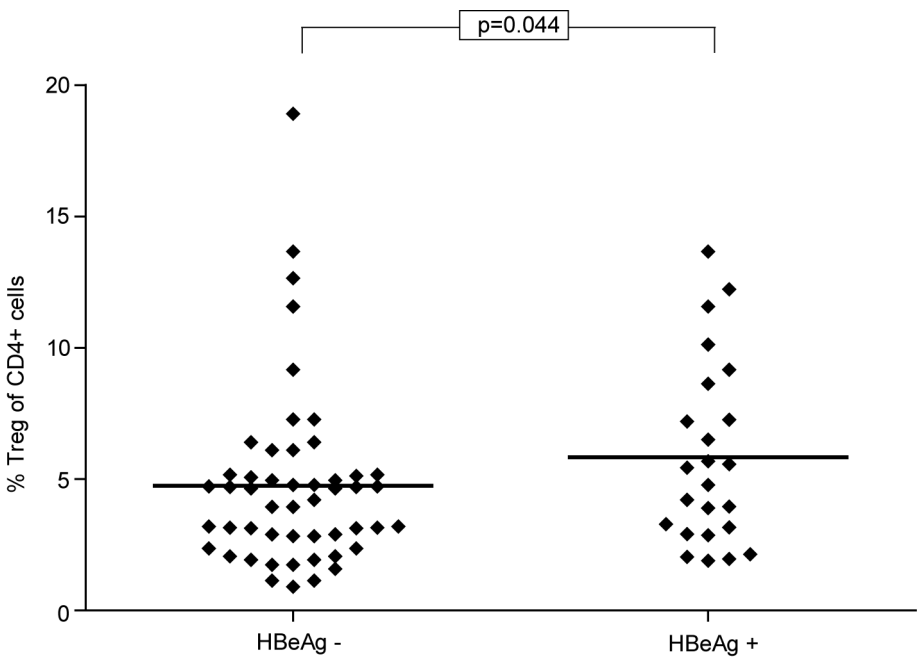

Figure 6. Patients who are HBeAg negative (n=21) have a lower percentage of Treg within the $CD4^+$ cell fraction in their peripheral blood compared to HBeAg-positive patients (n=24). The line represents the median percentage of Treg.

Discussion

The factors, which determine chronicity of HBV infection and the reason for the absence of a specific T-cell response are not clear. Negative selection, peripheral anergy and imbalances in lymphokine production all appear to contribute to maintaining the immunotolerant state (2). Recently, much attention has focussed on Treg as these cells have a prominent role in immunoregulation and tolerance (6-9). The present study demonstrates the importance of Treg in the persistence chronic HBV infection. We showed that patients with a chronic HBV infection contained a higher percentage of Treg in their peripheral blood compared to healthy controls and individuals with a resolved HBV infection. In addition, the relative *FoxP3* mRNA levels were higher in PBMC of patients with a chronic HBV infection. *FoxP3* is a transcription factor specifically expressed by $CD4^+CD25^+$ Treg (10,18-20). The higher relative level of *FoxP3* mRNA per $CD4^+$ cell indicates that chronic HBV patients have indeed a higher percentage of Treg within their population of $CD4^+$ cells in peripheral blood.

In our study we observed an association between the presence of serum HBeAg and an increased percentage of Treg, suggesting that HBeAg might be involved in the induction of Treg. This is supported by the observation that HBeAg can elicit T cell tolerance in murine experimental studies (21,22). No correlation was found between HBV DNA levels or alanine aminotransferase and the percentage of Treg. One could argue whether peripheral blood is the most appropriate compartment in HBV infections to assess accurately the presence of Treg and the relation to viral load and liver inflammation. Other studies have shown that Treg accumulate and expand locally at the site of infection, where they exert their suppressive activity (23-26). Therefore, we are currently assessing whether the relation between percentage of intrahepatic Treg and the viral load or liver inflammation is more prominent.

Several studies have demonstrated that the cellular immune response to HBcAg is generally low to undetectable in chronic HBV patients (27-31). This immune response may be suppressed by the increased presence of Treg found in these patients. Indeed, we showed that depletion of $CD4^+CD25^+$ cells clearly increased the HBcAg-specific T cell response in 17 patients. The effector cells suppressed by Treg are mainly $CD4^+$ T cells, since HBcAg has been described to induce predominantly a $CD4^+$ T cell response (27). Furthermore, when the isolated

Treg were reconstituted to the Treg-depleted fraction, the proliferation and the IFN-γ production were suppressed in a dose dependent manner.

In the present study depletion of $CD4^+CD25^+$ cells did not affect the tetanus toxin response, suggesting that the suppressive effect of Treg was HBV specific. Whether CD4+CD25+ cells inhibit the response to the recall antigen tetanus toxin, is still a matter of debate. In chronic *Helicobacter pylori* infection, CD25 depletion resulted in an increased proliferation against membrane proteins from the bacterium, while the tetanus response was unaffected (15). In two other studies $CD4^+CD25^+$ cells could inhibit the response against tetanus toxin (16,32).

In our experimental setting it is difficult to dissect the mechanism of immune suppression by Treg. In most *in vitro* models suppression is caused by a cell-cell contact dependent mechanism, yet the precise mechanism of suppression *in vivo* is not known (6,7,33). It was recently shown that Treg from patients with a chronic HCV infection produce IL10 and anti-TGF-β upon stimulation. The suppressive effect of the Treg was neutralized by addition of an anti-TGF-β antibody and experiments in a transwell culture system showed that the suppression was contact dependent (16). Further experiments will be necessary to determine the mechanism responsible for the suppression in an HBV specific experimental setting.

Although we cannot exclude that the increased percentage of Treg was pre-existent, predisposing the host to a chronic infection, the high number of Treg able to suppress HBV-specific T cell responses is most likely caused by the viral infection itself. It has been suggested that Treg can be induced through repetitive stimulation of T cells by high concentrations of antigen for long periods of time (17). Chronic HBV patients are known to have large amounts of HBV antigens in their peripheral blood. Results from studies of other chronic viral infections, such as cytomegalovirus, Human Immunodeficiency Virus and HCV have previously shown a role for Treg in the persistence of these infections (13,14). Depletion of $CD4^+CD25^+$ T cells from peripheral blood enhances T cell responses to cytomegalovirus and human immunodeficiency virus antigens (13). In patients with a chronic HCV infection a higher percentage of Treg was detected in peripheral blood compared to healthy controls and compared to individuals with a resolved HCV infection. The $CD4^+CD25^+$ Treg could directly suppress HCV-specific $CD8^+$ T cell IFN-γ production (14).

Treg can be induced by immature dendritic cells (DC) (34). Recently our group showed that myeloid DC isolated from chronic HBV patients were impaired in their

maturation and less capable of stimulating T cells compared to DC isolated from healthy individuals (35). Increased numbers of Treg are capable of inhibiting the maturation of DC and other antigen presenting cells, resulting in a decreased T cell stimulatory capacity (33, 36). This process could lead to the generation of more Treg and immature DC through a self-maintaining regulatory loop, which propagates HBV tolerance and chronicity of disease. An additional factor which may contribute to the increased percentage of Treg is the tolerogenic environment of the liver (37), the primary site of HBV replication.

In conclusion, patients with a chronic HBV infection have a higher percentage of Treg in their peripheral blood compared to healthy controls and individuals with a resolved HBV infection. These Treg are capable of inhibiting the HBV-specific immune response and this could thus contribute to persistence of HBV infection.

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CD4+CD25+ Regulatory T Cells decrease the upregulation of co-stimulatory molecules on dendritic cells during maturation

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Abstract

CD4+CD25+ regulatory T cells (Treg) can inhibit T cell responses. Their precise mechanism of suppression is not known. Treg can inhibit the T cell response in a direct manner, however it is suggested that they can also suppress the maturation of antigen presenting cells. In this study we have used the MUTZ-3 cell line as a source for dendritic cells (DC). The CD34+ human acute myeloid leukemia cell line MUTZ-3 possesses the unique ability to acquire a DC phenotype upon cytokine stimulation. The maturation capacity of MUTZ-3 derived immature DC, as determined by the expression of CD80, CD83 and CD86, was studied during co-culture with Treg. Co-culture of MUTZ-3 derived immature DC with CD4+CD25+Treg resulted in down regulation of CD80 and CD86, as compared to MUTZ-3 derived DC cultured alone or with CD4+CD25- T cells.

In conclusion, Treg can down regulate the expression of co-stimulatory molecules on DC. This reduced expression will lead to an impaired stimulatory capacity of DC.

Introduction

Regulatory T cells (Treg) are a subpopulation of CD4+ T cells capable of inhibiting the effector functions of $CD4^+$, $CD8^+$ and natural killer T cells (1-4). Several studies have shown that Treg play an important role in maintaining the peripheral immune tolerance (4,5). Treg have continuous expression of CD25 (the IL-2 receptor α-chain) and express the forkhead/ winged helix transcription factor gene (*FoxP3*). Retroviral gene transfer of *FoxP3* converts naïve T cells into $CD4^+CD25^+$ Treg capable of suppressing the response of other $CD4^+$ T cells (6).

Increasing evidence has become available showing that $CD4^+CD25^+$ Treg contribute to the immunological hypo-responsiveness against several pathogens, resulting in chronic infections. (7-11). Although there is evidence that Treg can inhibit the T cell response in an antigen presenting cell (APC) independent manner, an effect of Treg on the APC can not be excluded (12,13).

Dendritic cells (DC) represent the most potent APC and thus play an important role in the induction of specific T cell responses (14). Patients with a chronic Hepatitis B virus (HBV) or Hepatitis C virus (HCV) infection have peripheral DC that are impaired in their maturation and function (15,16). In addition, increased proportions of Treg have been described in patients chronically infected with one of these viruses (8,9,17). There is evidence that Treg have an effect on the function of monocytes and macrophages (18). If Treg are capable of inhibiting the T cell response by inhibiting APC function of DC, Treg might be the cause of the impaired DC functioning in chronically infected patients.

The MUTZ-3 cell line is a CD34+ human acute myeloid leukemia cell line, which behaves as the immortalized equivalent of CD34+ DC precursors. Immature DC can be generated from MUTZ-3 precursor cells by culturing the cells in culture medium supplemented with GM-CSF, IL4 and TNF-α. These immature DC can be stimulated to mature by increasing the concentration of TNF-α in the culture medium. Upon maturation the expression of HLA-DR, CD80, CD83 and CD86 on the DC increases. MUTZ-3 derived DC are capable of processing and presenting antigen and they are capable of stimulating T cells in an antigen specific and an allogenic setting (19). The Mutz-3 cell line is therefore an excellent source to obtain large numbers of dendritic cells.

In the present study we examined the effect of Treg on the maturation MUTZ-3 derived DC.

Materials en methods

Differentiation of Mutz-3 cells into immature. DC Differentiation of the precursor DC cell line was induced by supplementing the culture medium with GM-CSF, IL-4 and TNF-α. Minimum Essential Media alpha (Gibco, Paisley, UK) containing 20% heat-inactivated Fetal Calf Serum (FCS; Hyclone, Logan, UT), 100 U/ml Penicillin (Gibco), 100 µg/ml Streptomycin (Gibco, Paisley, UK), 50 µM β-mercaptoethanol was supplemented with 250 U/ml GM-CSF (Leucomax, Novartis Pharma, Arnhem, The Netherlands), 500 U/ml IL-4 (Strathmann Biotech) and 2,5 ng/ml TNF-α. After 6 days of differentiation the immature DC were phenotypically characterized by flowcytometry.

Isolation of $CD4^+CD25^+$ T cells and CD4+CD25- T cells. PBMC were obtained from heparinized peripheral blood samples of healthy controls (n=9) by ficoll separation (Ficoll-Paque™ plus, Amersham Biosciences, Buckinghamshire, UK). $CD4^+$ T cells were isolated from PBMC by negative selection using the untouched $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+CD25^+$ T cells were isolated from $CD4^+$ T cells using anti CD25-microbeads (Miltenyi biotec). The isolations were performed according to manufacturer's instructions. Purity of the cell fractions was determined by flowcytometry analysis with antibodies against CD4 and CD25. The following antibodies were used: anti-CD4-PerCP-Cy5.5 (SK3) (Becton Dickinson, San Jose, CA) and anti-CD25-PE (M-A251) (Pharmingen, San Diego, CA). An isotype matched control antibody was used to determine the level of background staining for the CD25 antibody. The $CD4^+CD25^+$ Treg purification method resulted in a Treg fraction containing 90% pure $CD4^+CD25^+$ Treg and a responder T cell fraction containing 90% pure CD4+CD25- cells.

Maturation of Mutz-3 derived Immature DC. Maturation of the MUTZ-3 cell line was performed in Minimum Essential Media alpha (Gibco, Paisley, UK) containing 20% heat-inactivated Fetal Calf Serum (FCS; Hyclone, Logan, UT),

100 U/ml Penicillin (Gibco), 100 µg/ml Streptomycin (Gibco, Paisley, UK), 50 µM β-mercaptoethanol supplemented with 250 U/ml GM-CSF (Leucomax, Novartis Pharma, Arnhem, The Netherlands), 500 U/ml IL-4 (Strathmann Biotech) and 75 ng/ml TNF-α. To $1x10^5$ immature DC, $1x10^5$ $CD4^+CD25^+$ Treg or $1x10^5$ CD4+CD25- responder T cells or a mixture of $1x10^5$ Treg and $1x10^5$ responder T cells were added. The cells were stimulated with anti-CD3 antibody (1 µg/ml) and anti-CD28 antibody (1 µg/ml). After 3 days of maturation the DC phenotype was determined by flowcytometry.

Phenotypic characterization of MUTZ-3 derived DC by flowcytometry. MUTZ-3 DC were incubated for 10 minutes with octagam (Octapharma, Lachen, Switzerland) in phosphate-buffered saline (PBS) containing 0,3% bovine serum albumin (BSA) before adding the antibodies. Cells were incubated for 30 minutes at 4ºC with antibodies. The following antibodies were used: anti-BDCA1 (CD1c)-PE (Miltenyi Biotec), anti-CD14-PerCP (MϕP9) (Becton Dickinson), anti-HLA-DR-FITC (L243) (Becton Dickinson), anti-CD83-APC (K562) (Becton Dickinson), anti-CD80-FITC (MAB104) (Immunotech), anti-CD86-APC (2331 FUN-1) (Becton Dickinson). Isotype-matched control antibodies were used to determine the level of background staining. After staining the cells were analyzed with a four-color cytometer (FACScalibur; CELL Quest Pro software, Beckton Dickinson).

Statistical analysis. Data are depicted as mean ± SEM. Flowcytometry data were analyzed with the Wilcoxon matched pairs signed rank sum test. For these analyses SPSS 11.5 for Windows (SPSS, Chicago, IL) was used.

Results

Co-culture of DC with Treg results in reduced upregulation of CD83. To determine the effect of Treg on DC maturation, the Mutz-3 derived immature DC were maturated without additional T cells, CD4+CD25- cells (responder T cells), CD4+CD25+ (Treg) or a mixture of CD4+CD25+ cells and CD4+CD25- T cells. The expression of the different markers on the DC was determined by flowcytometry. Since Mutz-3 cells can also differentiate into monocytes, DC were defined as the cells

that stained positive for BDCA-1 and negative for CD14. The presence of allogenic T cells, Treg as well as responder T cells, resulted in an increased proportion of Mutz-3 derived DC expressing CD83 (78.3% ± 8.7% (no T cells), 90.7% ± 8.7% (Treg), 92.8% ± 3.1% (responder T cells) and 90.0% ± 3.7% (Treg and responder T cells)). The same difference in upregulation was observed for the expression level of CD83 on the dendritic cells, as indicated by the mean fluorescence intensity (279.0 ± 32.6 (no T cells), 421.5 ± 45.8 (Treg), 451.8 ± 37.3 (responder T cells) and 421.4 ± 36.2 (Treg and responder T cells); Figure 1).

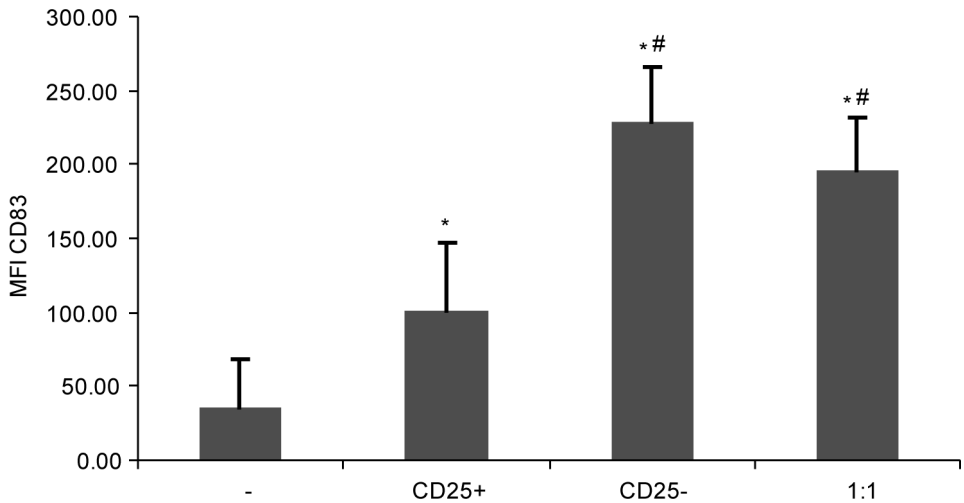

Figure 1. Mutz-3 derived DC were maturated alone (-), in the presence of Treg (CD25+), responder T cells (CD25-) or in the presence of responder T cells and Treg (1:1). CD83 expression of DC was determined by flowcytometry. DC are defined as BDCA-1 positive and CD14 negative cells. The mean fluorescence intensity (MFI) of CD83 on the dendritic cells. ∗ $p < 0.05$ compared to no T cells, # $p < 0.05$ compared to DC cultured with Treg, ¶ $p < 0.05$ compared to DC cultured with responder T cells (n = 9).

Treg had no effect on the HLA-DR expression of DC. The expression of HLA-DR and co-stimulatory molecules by DC is essential for the antigen presenting function. Therefore the effect of Treg on HLA-DR expression was determined. Treg had no significant effect on the expression levels of HLA-DR by Mutz-3 derived DC. The proportion of BDCA-1 positive cells expressing HLA-DR was more than 90% for all culture conditions. The expression level of HLA-DR by BDCA-1 positive cells as indicated by the mean fluorescence intensity was 680.1 ± 184.5 (no T cells),

609.5 ± 207 (Treg), 793.2 ± 182 (responder T cells) and 732.0 ± 199.6 (Treg and responder T cells).

Treg inhibit the up-regulation of co-stimulatory molecules by DC. Co-culture of MUTZ-3 derived immature DC with Treg resulted in a significantly smaller proportion of Mutz-3 derived DC expressing CD80 (70.9% ± 4.2% (no T cells), 56.1% ± 4.48% (Treg; $p < 0.05$ compared to no T cells), 71.1% ± 3.0% (responder T cells) and 70.1% ± 3.8% (Treg and responder T cells). When analyzing the expression level of the CD80 per DC (as indicated by the mean fluorescence intensity) co-culture of DC with Treg also resulted in a significantly less upregulated expression (86.5 ± 41.5 (no T cells), 42.9 ± 13.8 (Treg), 81.6 ± 14.6 (responder T cells) and 71.8 ± 12.6 (Treg and responder T cells); Figure 2).

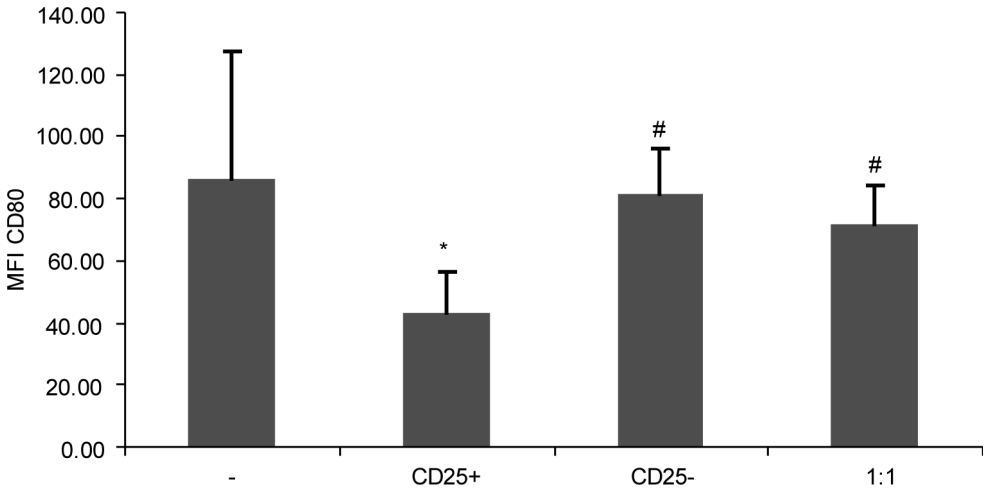

Figure 2. Mutz-3 derived DC were maturated alone (-), in the presence of Treg (CD25+), responder T cells (CD25-) or in the presence of responder T cells and Treg (1:1). CD80 expression of DC was determined by flowcytometry DC are defined as BDCA-1 positive and CD14 negative cells. The mean fluorescence intensity (MFI) of CD80 on the dendritic cells. ∗ $p < 0.05$ compared to no T cells, # $p < 0.05$ compared to DC cultured with Treg, ¶ $p < 0.05$ compared to DC cultured with responder T cells ($n = 9$).

In addition, the presence of Treg during maturation significantly affected the proportion of Mutz-3 derived DC expressing CD86 (92.9% ± 1.4% (no T cells), 88.0% ± 2.5% (Treg; $p < 0.05$ compared to no T cells), 91.2% ± 1.3% (responder T cells) and 90.7% ± 1.2% (Treg and responder T cells)). The mean fluorescence intensity

for CD86 showed the same reduced upregulation after co-culture with Treg (715.4 ± 89.3 (no T cells), 478.0 ± 367.6 (Treg), 907.7 ± 86.9 (responder T cells) and 680.4 ± 89.2 (Treg and responder T cells); Figure 3).

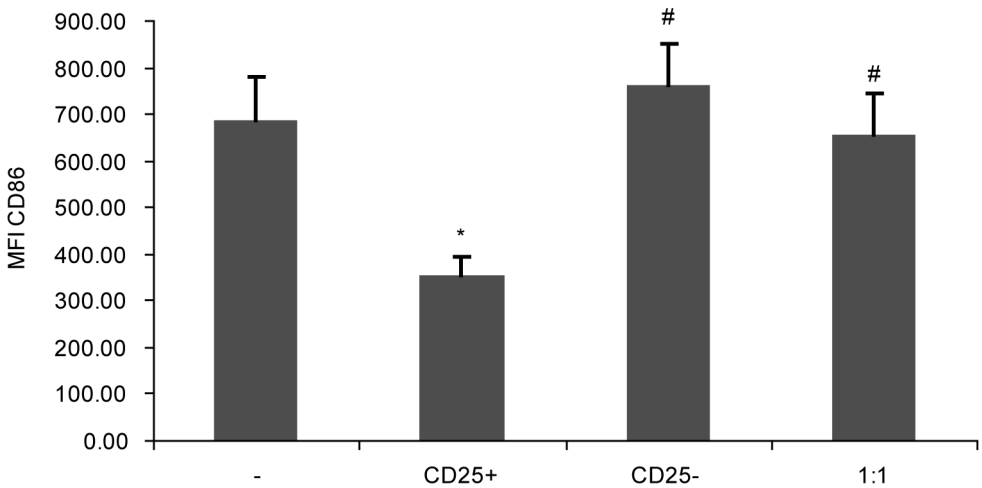

Figure 3. Mutz-3 derived DC were maturated alone (-), in the presence of Treg (CD25+), responder T cells (CD25-) or in the presence of responder T cells and Treg (1:1). CD86 expression of DC was determined by flowcytometry DC are defined as BDCA-1 positive and CD14 negative cells. The mean fluorescence intensity (MFI) of CD86 on the dendritic cells. ∗ $p < 0.05$ compared to no T cells, # $p < 0.05$ compared to DC cultured with Treg, ¶ $p < 0.05$ compared to DC cultured with responder T cells (n = 9).

Discussion

Treg are a subpopulation of CD4+ T cells capable of inhibiting the effector functions of $CD4^+$, $CD8^+$ and natural killer T cells (1-4). Several studies have shown that Treg are essential for maintaining peripheral immune tolerance (4,5). There is increasing evidence that Treg also contribute to the immunological hypo-responsiveness against several pathogens, resulting in chronic infections (7-11). Although there is evidence that Treg can inhibit the T cell response in an APC independent manner, an effect of Treg on the APC can not be excluded (12,13).

The present study demonstrates that Treg can inhibit the maturation of DC. In an allo-response setting the co-culture of DC with Treg resulted in down regulation of the maturation markers CD80 and CD86 compared with co-culture of DC with responder T cells or culture with no additional T cells. The findings for CD80

and CD86 are consistent with those found in earlier studies where blood derived CD1a positive DC and monocytes were used (18,20). The presence of Treg during maturation did not result in less upregulation of CD83 and HLA-DR compared to Treg maturated without any T cells present. Houot *et al* had similar findings. In addition to a lack of effect on HLA-DR and CD83, they also showed that CD40 upregulation was not affected by Treg and that Treg had no effect on plasmacytoid DC (21). In all these studies DC were functionally impaired after co-culture with Treg (18 20,21).

The upregulation of CD83, which is seen after co-culture with T cells, might be due to either a stimulatory signal provided by CD40L on the T cells or to IFN-γ produced by the responder T cells. As described, IFN-γ increases the expression of maturation markers on DC and thus increases their stimulatory capacity (22).

No effect on DC maturation was observed when T cells were cultured with DC without anti-CD3 and anti-CD28 present (data not shown). This could be due to the low expression of co-stimulatory molecules on Mutz-3-derived DC, which enabled them to activate the T cells. Nevertheless, in other studies where DC and Treg were co-cultured in the presence of anti-CD3 and anti CD28 , at least one study shows that allogenic stimulus of the immature DC was sufficient to activate T cells (23). No significant difference was observed between DC co-cultured with responder T cells and DC co-cultured with responder T cells and Treg. The most likely explanation for these findings is that either the Treg were not capable of significantly inhibiting responder T cell function or that responder T cells had a more potent effect on DC compared to Treg.

Earlier studies have shown that these patients have increased percentages of Treg in their peripheral blood and that they have functionally impaired DC (8,9, 15,16). The effect that Treg have on DC maturation could be an explanation for the impaired DC function which is observed in patients with chronic Hepatitis B and Hepatitis C infections. Treg can be induced by immature DC (24). We and others have shown that Treg are capable of inhibiting the maturation of DC and other antigen presenting cells, resulting in a decreased T cell stimulatory capacity (12,20). This process could lead to the generation of more Treg and immature DC through a self-maintaining regulatory loop, which propagates tolerance and chronicity of disease in for example HBV and HCV.

In conclusion, Treg can inhibit the upregulation of the co-stimulatory molecules CD80 and CD86 during DC maturation. This inhibition by Treg leads to functionally impaired DC due to a diminished co-stimulatory capacity.

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Tumor necrosis factor-α inhibits the suppressive effect of regulatory T cells on the HBV-specific immune response

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Abstract

Chronicity of hepatitis B virus (HBV) infection is characterized by a weak immune response to the virus. $CD4^+CD25^+$ regulatory T cells (Treg) are present in increased numbers in the peripheral blood of chronic HBV patients and these Treg are capable of suppressing the HBV-specific immune response. The aim of this study was to abrogate Treg mediated suppression of the HBV-specific immune response. Therefore, Treg and a Treg depleted cell fraction were isolated from peripheral blood of chronic HBV patients. Subsequently, the suppressive effect of Treg on the response to HBV core antigen (HBcAg) and tetanus toxin was compared and the effect of exogenous tumor necrosis factor (TNF)-α, IL-1β or neutralizing antibodies against IL-10 or TGF-B on Treg mediated suppression was determined. The results show that Treg of chronic HBV patients had a more potent suppressive effect on the response to HBcAg compared to the response to tetanus toxin. Neutralization of IL-10 and TGF-B or exogenous IL-1β had no effect on Treg mediated suppression of the anti-HBcAg response, while exogenous TNF-α partially abrogated Treg mediated suppression. Pre-incubation of Treg with TNF-α demonstrated that TNF-α had a direct effect on the Treg. No difference was observed in the type II TNF receptor expression by Treg from chronic HBV patients and healthy controls.

In conclusion, Treg mediated suppression of the anti-HBV response can be reduced by exogenous TNF-α. Since chronic HBV patients are known to produce less TNF-α, these data implicate an important role for TNF-α in the impaired anti-viral response in chronic HBV.

Introduction

Worldwide 400 million people suffer from a chronic hepatitis B virus (HBV) infection and approximately 1 million people die annually from HBV-related disease. Infection with HBV in adults results frequently in a self-limiting, acute hepatitis, which confers protective immunity and causes no further disease. In 10% of infected adults HBV leads to a chronic infection (1-3). In patients with an acute self limiting HBV infection, a multispecific $CD4^+$ and $CD8^+$ T cell response with a type 1 cytokine profile, is important for control of the infection (4,5). Patients with a chronic HBV infection lack such a vigorous multispecific T cell response (6,7).

Regulatory T cells (Treg) play an important role in maintaining the peripheral immune tolerance (8-10). Treg can be distinguished from $CD4^+$ effector T cells by their continuous expression of the activation marker CD25 and the expression of the Treg specific transcription factor FoxP3 (11,12). Besides regulating peripheral tolerance, Treg can also affect T cell responses to foreign antigens (13). Patients with a chronic HBV infection have increased percentages of Treg in their peripheral blood compared to healthy controls and individuals who have resolved their HBV infection (14). Recently, Xu *et al* showed that patients with a high viral load have an increased proportion of Treg compared to patients with a low viral load (15). Treg from patients with a chronic HBV infection are capable of inhibiting the HBV specific $CD4^+$ and $CD8^+$ T cell response in a dose dependent manner (14-16).

The mechanism of the suppressive activity exerted by Treg on the T cell response remains unclear. There is inconsistency in the literature about the possible involvement of the immunoregulatory cytokines IL-10 and TGF-β (17,18). Recently, it was shown that Treg express the type II TNF receptor (19). Tumor necrosis factor (TNF)-α plays an important role in the control of viral infections. It is involved in the recruitment and activation of macrophages, it can polarize the T cell response towards the development of antiviral effector functions and it has a direct antiviral effect (20,21). TNF-α is essential for the proliferation of HBV-specific cytotoxic T cells (22).

Given the important role of Treg in the impaired anti-viral immune response during a chronic HBV infection, this study is focused on which factors can abrogate Treg mediated suppression and restore the anti-HBV immune response.

Materials and Methods

Patients. For functional experiments peripheral blood samples were obtained from chronic HBV patients. All patients had detectable HBVDNA levels in serum. For the functional assays PBMC were used from 29 patients with a chronic HBV infection. Eleven patients were HBV e antigen (HBeAg) positive, the median viral load was 2.5 x 10^6 geq/ml (1.0 x 10^3- 1.6 x 10^{10}) and the median ALT level was 32 units/l (16-321). For TNF receptor staining peripheral blood samples from 13 age matched chronic HBV patients and healthy controls were used. Six patients were HBeAg positive, the median viral load was 3.5 x 10^5 geq/ml (1.0 x 10^3- 1.6 x 10^{10}) and the median ALT level was 32 units/l (11-137). Patients co-infected with either human immunodeficiency virus, hepatitis A virus, hepatitis C virus or hepatitis D virus and patients with a resolved viral hepatitis were excluded from this study. Also patients and controls who were immunocompromised or pregnant and patients that received antiviral or immunomodulatory HBV treatment during the last 6 months before blood sampling were excluded from this study. The institutional review board of the Erasmus MC – University Medical Center Rotterdam, approved this study, and informed consent was obtained from all patients prior to their inclusion in this study.

$CD4^+CD25^+$ T Cell isolation. PBMC from chronic HBV patients were obtained by ficoll separation (Ficoll-Paque™ plus, Amersham Biosciences, Buckinghamshire, UK). $CD4^+$ T cells were isolated from PBMC by negative selection using the untouched $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+CD25^+$ T cells were isolated from $CD4^+$ T cells using anti CD25-microbeads (Miltenyi Biotec). The isolations were performed according to manufacturer's instructions. The $CD4^-$ and the $CD4^+CD25^-$ fraction were pooled and were used as CD25 (Treg)-depleted responder cells. The $CD4^+CD25^+$ Treg purification method resulted in a Treg fraction containing more than 90% pure $CD4^+CD25^+$ Treg and a Treg-depleted cell fraction containing all other cell types present in PBMC. These isolated cell fractions were used in proliferation assays. Depending on the yield of isolated $CD4^+CD25^+$ cells, the cells were used for the different functional experiments.

Flowcytometry. The purity of the isolated cell fractions was determined by flowcytometry with antibodies against CD4 and CD25. The following antibodies were used, anti-CD4-PerCP-Cy5.5 (SK3) (Becton Dickinson, San Jose, CA) and anti-CD25-PE (M-A251) (BD Pharmingen, San Jose, CA). An isotype matched control antibody was used to determine the level of background staining for the CD25-PE antibody. The viability of Treg, pre-incubated with either IL-2 alone or IL-2 and TNF-α, was determined using 7AAD (BD Pharmingen) and annexin V-APC (BD Pharmingen).

The FoxP3 antibody staining was performed according the manufacturer's instructions. Briefly, cell surface markers were stained with anti-CD25-PE and anti-CD4-PerCP-Cy5.5. The cells were fixed and permeabilized with Fix/perm buffer and permeabilization buffer (eBiosciences, San Diego, Ca) anti-FoxP3-APC (PCH101) (eBiosciences) was added during permeabilization. For the CD120b (TNF receptor type II) staining, biotin conjugated anti-CD120b (hTNFR-M1) (BD Pharmingen) and streptavidin-PerCP (Becton Dickinson), anti-CD25-PE, anti-FoxP3-APC and anti CD4-FITC (13B8.2; Immunotech, Marseille, France) were used. For anti-CD25-PE, anti-FoxP3-APC and biotin conjugated anti-CD120b isotype matched control antibodies were used. After staining the cells were analyzed using a four-color cytometer (FACScalibur^{TM}, CELLQuest Pro^{TM} software, Beckton Dickinson).

Proliferation assay. The proliferation of $CD4^+$ T cells was determined as described earlier (14). Briefly, the pooled CD25-depleted cells and the pooled CD25-depleted cells supplemented with 20% $CD4^+CD25^+$ Treg were cultured in triplicate. CD25-depleted cells were cultured in a concentration of $1X10^5$ cells per well in 100 µl RPMI 1640 (Bio Whittaker, Verviers, Belgium) containing 5% pooled human serum (Dept. of Immunohematology and Bloodbank, Leiden University Medical Center, Leiden, The Netherlands) and penicillin/streptomycin (Gibco, Paisley, UK). For the culture condition with 20% Treg, 25,000 of the isolated $CD4^+CD25^+$ cells were added to the Treg depleted cells. The cells were cultured for six days either in the presence of 1 µg/ml HBV core antigen (HBcAg) (a kind gift by M. van Roosmalen, Biomerieux, Boxtel, The Netherlands), 15 limits of flocculation/ml purified tetanus toxin (SVM, Bilthoven, The Netherlands) or without extra stimuli. For IL-10 neutralization, 5 µg/ml rat anti-human and viral IL-10 (JES-9D7) (BD Pharmingen) was used and Rat IgG1 (R3-34) (BD Pharmingen) was used as an isotype matched control antibody. For

TGF-β neutralization, 0.5 µg/ml goat anti Human TGF-β1 (anti-human LAP (TGF-β1); R&D systems, Oxon, United Kingdom) was used and purified normal goat IgG (R&D systems) was used as an isotype matched control antibody. To determine the effect on TNF-α and IL-1β on Treg mediated suppression, 25 ng/ml TNF-α (Strathman Biotech) or 50 ng/ml IL-1β (Strathman Biotech) was added to the cell culture medium. For the Treg pre-incubation experiment, the CD4+CD25+ were cultured overnight with 25 ng/ml TNF-α and 100 u/ml IL-2 (Chiron corporation) or with IL-2 alone. After this overnight incubation, the Treg were washed twice with RPMI 1640 containing 5% human serum and added to HBcAg stimulated CD25-depleted cells.

After 5 days of incubation the cells were pulsed with 0.25 µCi/well of [^{3}H]-thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK). The cells were harvested 16 hours later. Proliferation was determined by liquid scintillation counting of the harvested cells and expressed as stimulation index (SI).

Mathematics and statistics. All results are given as mean ± SEM. The SI was calculated from the counts per minute with antigen divided by the counts per minute without antigen. The percentage of suppression by Treg was determined using the following formula: (1- S.I. (Treg depleted cells + 20% Treg) / S.I. (Treg depleted cells)) x 100%. The data were analyzed with SPSS11.5 for Windows (SPSS, Chicago, IL). The Wilcoxon paired signed rank sum test was used to analyze the data from the experiments in which the % of suppression between two different culture conditions was compared.

Results

Treg from HBV patients have a stronger suppressive effect on the HBV-specific response compared to the response against tetanus toxin. Treg were isolated using a CD4+ and CD25+ isolation kit, subsequently the FoxP3 expression of the isolated $CD4^+CD25^+$ cells (Treg) and the CD25 (Treg) depleted cell fraction was determined. To determine whether the suppression of the response against HBV by Treg was antigen specific, the suppressive effect of Treg on the response against tetanus toxin and HBcAg was compared. Data from one representative patient are shown in figure 1A. Treg showed a stronger inhibitory effect on the response against HBcAg compared to the response against tetanus toxin (59.7% ± 4.3% vs. 40.8%

± 6.7%, p= 0.02; figure 1B). No correlation was observed between the suppressive capacity of Treg and viral load or ALT levels of the patients. Also the suppressive capacity between Treg isolated from HBeAg positive or HBeAg negative patients was not different (data not shown).

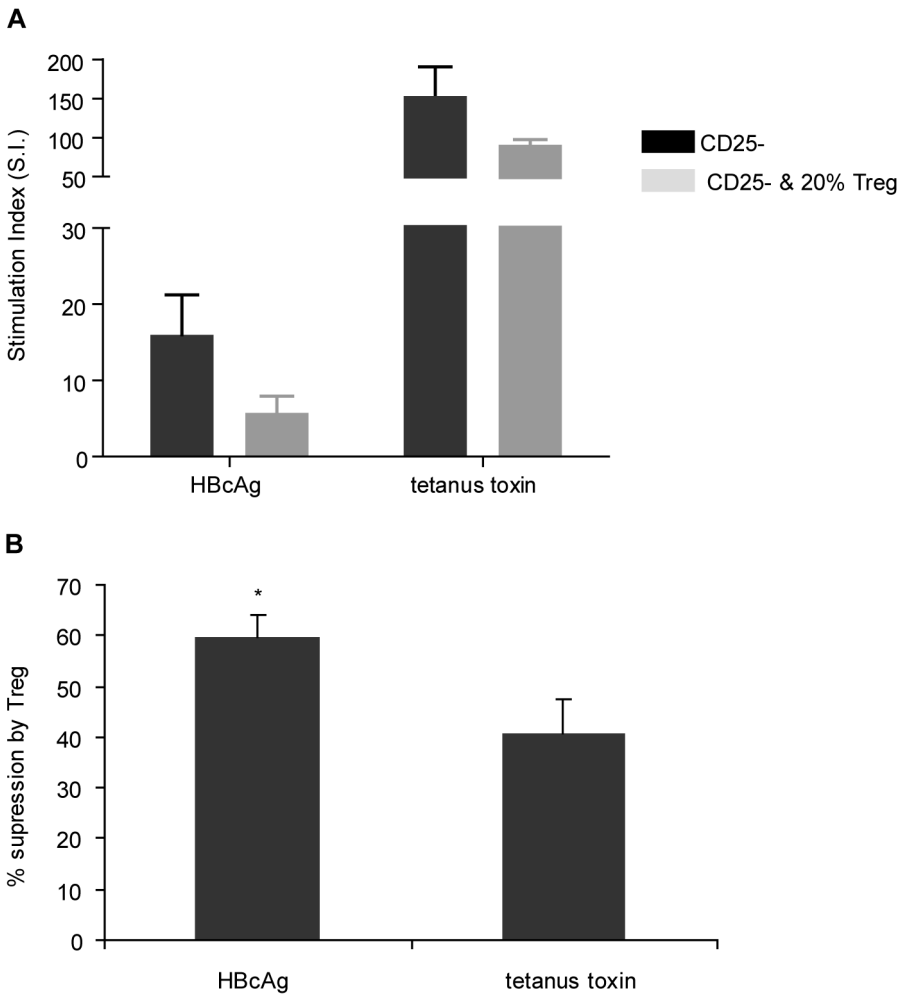

Figure 1. Treg depleted cells and Treg depleted cells reconstituted with 20% $CD4^+CD25^+$ Treg were stimulated with either HBcAg or with tetanus toxin. After 6 days the proliferation was determined by [^{3}H]-thymidine incorporation. The proliferation is depicted as stimulation index (S.I.; counts stimulated cells/ counts unstimulated cells). **(A)** S.I. of a representative experiment with cells from an HBV patient. The black bars are Treg depleted cells and the grey bars are Treg depleted cells reconstituted with 20% of Treg. **(B)** The mean percentage of suppression by 20% of Treg on the response to HBcAg and tetanus toxin (n = 18). The percentage of suppression was calculated using the following formula: (1- S.I. (Treg depleted cells + 20% Treg) / S.I. (Treg depleted cells)) x 100%. * $p < 0.05$ compared to the tetanus toxin response.

Neutralization of IL-10 or TGF-β does not abrogate Treg mediated suppression. Activated Treg can produce the immunoregulatory cytokines IL-10 and TGF-β, however it is unclear whether these cells exhibit their suppression via these cytokines. Therefore we determined the effect of neutralization of IL-10 and TGF-β on HBV-specific Treg mediated suppression for 10 patients. Treg depleted cells and Treg depleted cells reconstituted with 20% Treg were stimulated with HBcAg in the presence of neutralizing antibodies against IL-10 or TGF-β or isotype matched control antibodies. Neutralization of IL-10 did not affect Treg mediated suppression (48.5% ± 11.1% vs. 48.1% ± 11.7%). Also, neutralization of TGF-β did not result in a decrease of Treg mediated suppression of response against HBcAg (32.6% ± 12.3% vs. 29.8% ± 11.2%).

TNF-α partially abrogates Treg mediated suppression of the response against HBcAg. To determine whether pro-inflammatory cytokines have a modulatory effect on Treg mediated suppression of the response against HBcAg, Treg depleted cells and Treg depleted cells reconstituted with 20% of Treg were stimulated with HBcAg in the presence of exogenous TNF-α or IL-1β. Incubation with exogenous TNF-α resulted in a decrease in the Treg suppression of the proliferation (62.4% suppression ± 5.3% (no cytokine) vs. 29.8% ± 7.8% (TNF-α), p = 0.02). This effect was not observed when IL-1β was added to the proliferation assay (54.2% ± 8.7%) (Figure 2A). Since, the receptor for TNF is not only expressed by Treg but also by a variety of other cells, TNF-α could theoretically affect all cell types present in culture. Therefore, we determined whether the decreased suppressive capacity was caused by a direct effect of TNF-α on Treg. Isolated Treg were pre-incubated overnight with either IL-2 alone or with IL-2 and TNF-α. After overnight culture Treg were extensively washed and added to HBcAg stimulated Treg depleted cells. Figure 2B shows that pre-incubation of Treg with TNF-α and IL-2 resulted in a partial abrogation of Treg mediated suppression as well (42.0% suppression ± 6.0% (IL-2 alone) vs. 19.5% ± 7.4% (IL-2 and TNF-α), p = 0.016). In addition, TNF-α partially abrogated the suppressive effect of Treg from patients with a chronic HBV infection and healthy controls when tetanus toxin was used as a stimulus (data not shown).

To examine whether TNF-α affected the vitality of Treg, cells were pre-incubated with IL-2 alone or IL-2 and TNF-α. Their vitality after pre-incubation was determined by flowcytometry with 7AAD and Annexin V. Pre-incubation of Treg with TNF-α did not result in a decreased vitality, no increase in apoptosis and cell death

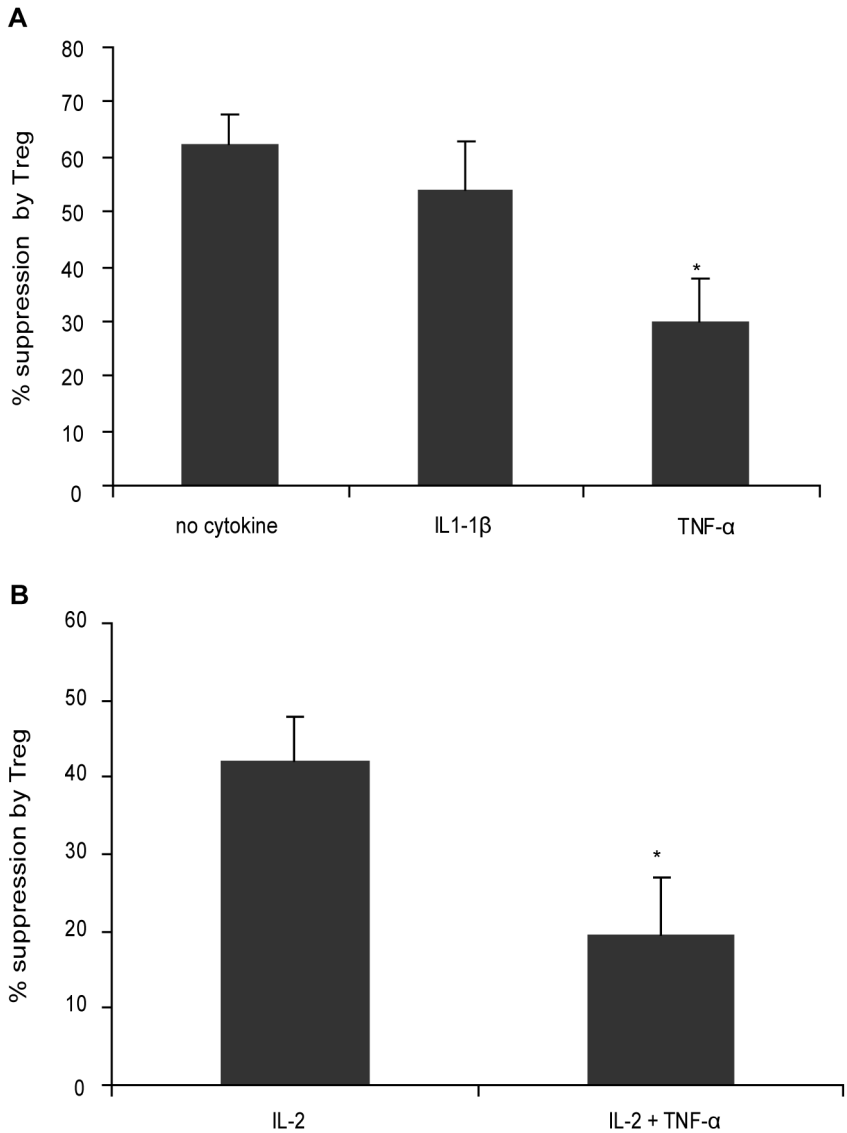

Figure 2. (A) Treg depleted cells and Treg depleted cells reconstituted with 20% CD4+CD25+Treg were stimulated with HBcAg in the presence of no additional cytokines 25 ng/ml TNF-α or 50 ng/ml IL-1β (n=10) **(B)** Treg depleted cells were stimulated with HBcAg and Treg were incubated o/n with either IL-2 or IL-2 and TNF-α (n=7). After overnight pre-incubation Treg were added to HBcAg stimulated Treg depleted cells. After 6 days the proliferation was determined by [^{3}H]-thymidine incorporation. The percentage of suppression was calculated using the following formula: (1- S.I. (Treg depleted cells + 20% Treg) / S.I. (Treg depleted cells)). Data are depicted as mean ± SEM.* $p < 0.05$ compared to control.

was observed (figure 3A and 3B). During co-culture there was also no difference in cell death observed between Treg pre-incubated with IL-2 and TNF-α or IL-2 alone as determined by flowcytometry with anti-FoxP3-APC and 7AAD (3.5% $\pm$ 0.8% vs. 3.7% $\pm$ 1.0%). Since it has been suggested that FoxP3 expression is related to the suppressive capacity of Treg, the effect of TNF-α on FoxP3 expression was determined. Overnight pre-incubation with TNF-α did not affect the FoxP3 expression of Treg (figure 4C and 4D). In addition, pre-incubation of Treg with TNF-α did result in a down regulation CD120b on Treg (Figure 4E and 4F, n=5; p = 0.04).

Similar expression of TNF receptor type II was observed by Treg of chronic HBV patients and healthy controls. TNF-α has a direct effect on Treg mediated suppression, therefore TNF receptor expression by Treg might be an indication of the Treg sensitivity for TNF-α of the Treg. The TNF type II receptor (CD120b) has been shown to be expressed by Treg (19). Therefore, freshly isolated PBMC from patients with a chronic HBV infection and healthy controls were stained with antibodies against CD120b, CD4 and FoxP3. No difference was observed in the expression of CD120b by $CD4^+FoxP3^+$ cells between patients and healthy controls. CD120b is expressed by 26.6% $\pm$ 3.6% $CD4^+FoxP3^+$ cells in patients vs. 26.8 % $\pm$ 3.5% in healthy controls (figure 4).

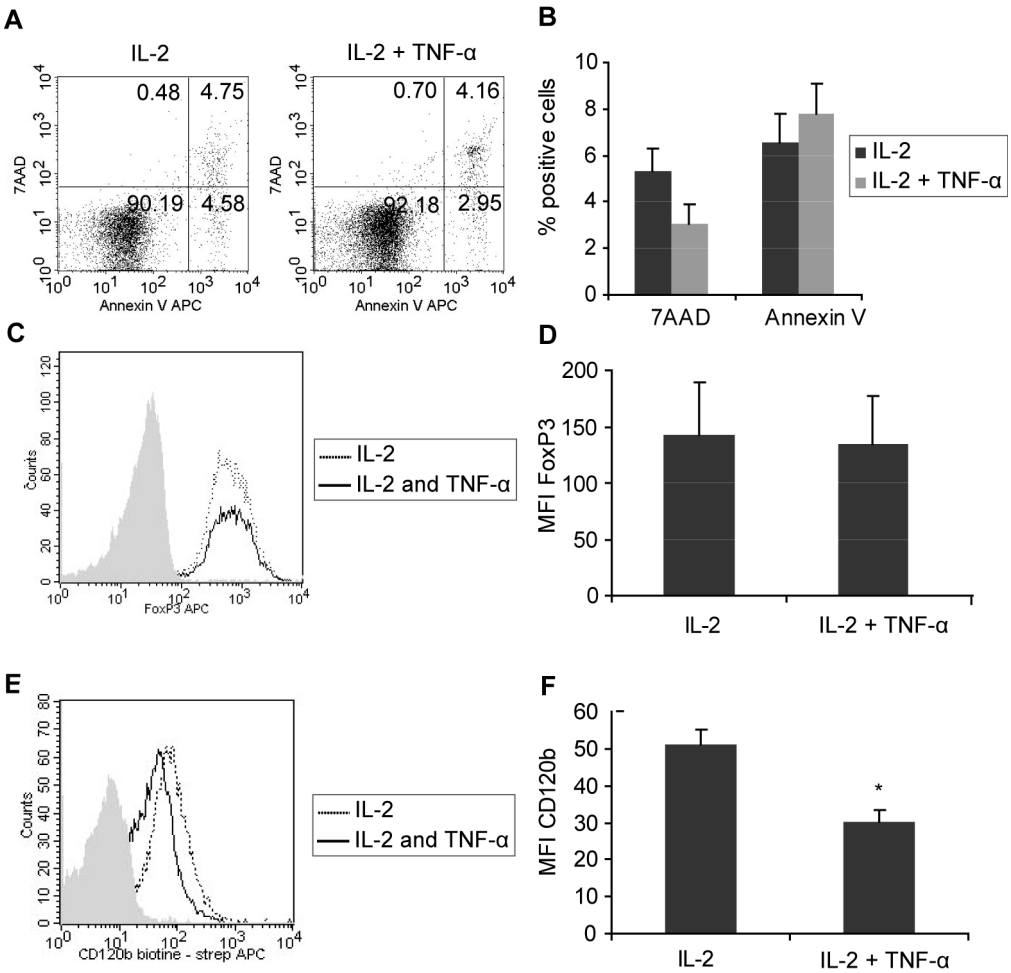

Figure 3. Treg were overnight incubated with either IL-2 or IL-2 and TNF-α (n=4). Cell death and apoptosis was determined by staining with 7AAD and Annexin V. **(A)** Flowcytometry data from one representative 7AAD and Annexin V staining. **(B)** Percentages death and apoptotic cells**.** Data are expressed as mean ± SEM. **(C)** Flowcytometry data from a representative FoxP3 staining after incubation with either IL-2 and TNF-α or IL-2 alone. The dotted line depicts the FoxP3 expression of cells pre-incubated with IL2 and the solid line depicts the FoxP3 expression of cells pre-incubated with IL-2 and TNF-α. The staining with the isotype matched control antibody is depicted in grey. **(D)** The mean fluorescence intensity (MFI) of FoxP3 of pre-incubated Treg. The data are expressed as mean ± SEM. **(E)** Representative staining for CD120b on Treg incubated with either IL-2 and TNF-α or IL-2 alone. The dotted line depicts the CD120b expression of cells pre-incubated with IL2 and the solid line depicts the CD120b expression of cells pre-incubated with IL-2 and TNF-α. The staining with the isotype matched control antibody is depicted in grey. **(F)** The mean fluorescence intensity (MFI) of CD120b of pre-incubated Treg. The data are expressed as mean ± SEM.* P< 0.05 compared to control.

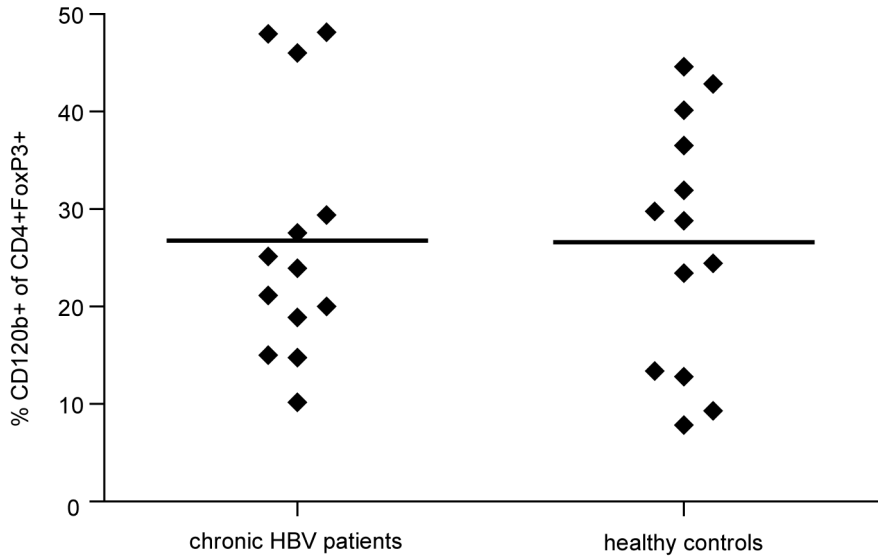

Figure 4. The proportion of TNF receptor type II (CD120b) positive Treg in patients and healthy controls determined by flowcytometry. Freshly isolated PBMC stained with antibodies against CD120b, FoxP3 and CD4. Treg were defined as cells staining positive for CD4 and FoxP3. The bar represents the mean proportion of Treg expressing CD120b (n=13).

Discussion

Treg are involved in the impaired immune response during chronic HBV. They are capable of inhibiting HBV-specific proliferation and IFN-γ production of $CD4^+$ and $CD8^+$ T cells (14-16). Treg can have a direct inhibitory effect on T cells or can inhibit the T cell response in an indirect manner by inhibiting dendritic cell (DC) maturation (17,23).

Treg have to be activated through their T cell receptor to become suppressive. After activation the suppression by Treg is non-antigen specific (24). Treg suppress the immune response in a dose dependent manner (25). The present study shows that the suppressive effect of circulating Treg, of a chronic HBV patient, on the CD4+ T cell response to HBcAg is proportionally more potent as compared to the suppressive effect on the response to tetanus toxin. This could explain the fact that chronic HBV patients have an impaired HBV specific immune response, but still have an adequate immune response to tetanus toxin. In Fact, the presence of HBV specific Treg has been described in a recent publication (26).

Treg can secrete IL-10 and TGF-β after activation (8,27,28), however it was not possible to abrogate Treg mediated suppression of the HBV-specific response by means of IL-10 or TGF-β neutralization. This concurs with data from earlier *in vitro* studies in which neutralization of these two cytokines had no effect on the suppression of T cells stimulated with anti CD3 and anti CD28 (25,29).

Our study shows that exogenous TNF-α can abrogate Treg mediated suppression. Pro inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, are important mediators during the initiation of an adaptive immune response. IL-6 can influence suppression by Treg, however not through a direct effect on Treg, but by making effector cells anergic for Treg mediated suppression (30). The present study shows that TNF-α was capable of directly affecting the suppressive capacity of Treg. IL-1β had no such effect. Treg can express the TNF receptor type II (19), however there was no difference in the expression of this receptor between Treg of patients with a chronic HBV infection and Treg of healthy controls. Incubation of Treg with TNF-α resulted in a down regulation of the type II TNF receptor by Treg. Furthermore, the inhibitory effect of TNF-α on Treg mediated suppression was not due to cell death of Treg, since pre-incubation of Treg with TNF-α did not result in increased cell death or apoptosis. It has been suggested that FoxP3 expression correlates with the suppressive capacity of Treg. Valencia *et al* showed that incubation of Treg with TNF decreased the FoxP3 expression of Treg and abrogated their suppressive capacity (19). In our study we showed that the TNF-induced abrogation of the Treg suppressive capacity was not mediated via down modulation of FoxP3 expression. No down regulation of FoxP3 expression by Treg was observed after overnight incubation or incubation for 48 hours (data not shown) with TNF-α. The cytokine environment and the availability of cytokine receptors are factors that can influence the immune response in chronic HBV patients. Neutralization of the TNF receptor during therapy with Infliximab for Crohn's disease, resulted in (re) activation of hepatitis B virus among chronic HBV carriers (31,32). It has recently been shown that Infliximab restores the suppressive capacity of Treg (19,33). PBMC from patients with an acute HBV infection produce more TNF-α compared to PBMC from patients with a chronic HBV infection and in a previous study by Van der Molen *et al* was shown that the total amount of TNF-α produced by isolated myeloid derived DC from patients with a chronic HBV infection was decreased compared to myeloid derived DC form healthy controls (34,35). The decreased TNF-α production observed in

patients with a chronic HBV infection could result in the presence of Treg that are more suppressive for the HBV-specific immune response.

TNF-α appears to be a key mediator in the chronicity of HBV, it can partially abrogate Treg mediated suppression of HBV specific T cells, is essential for the proliferation of HBV-specific cytotoxic T lymphocytes (22), has a direct anti-viral effect (20) and can induce DC maturation (36). Since, TNF-a is such an important cytokine during a chronic HBV infection, TNF-α might be a target for future immunotherapeutic strategies.

In conclusion, the suppression of the response against HBV by Treg is partially HBV specific and cannot be abrogated by neutralization of IL-10 and TGF-β or by exogenous IL-1β. TNF-α is capable of decreasing the suppressive capacity of Treg. Together with the decreased TNF-α production observed in patients with a chronic HBV infection, this suggests that TNF-α is an important mediator of the impaired immune response observed in patients with a chronic HBV infection.

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Higher percentages of intra-hepatic regulatory T cells are present in chronic hepatitis B patients with a high viral load

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Abstract

Chronic hepatitis B virus (HBV) infection is characterized by a weak immune response to HBV. Regulatory T cells (Treg) play a key role in this impaired immune response. Most studies concerning Treg and HBV are predominantly focused on peripheral blood Treg. It is however questionable whether these studies are representative for chronic HBV infection in humans, since the liver is the site of infection. In this study we have therefore monitored intra-hepatic Treg. We compared the proportional frequency of Treg in peripheral blood and the liver of 40 chronic HBV patients. A higher percentage of Treg, defined as CD4, CD25 and FoxP3 positive cells was detected in the liver of patients with a high viral load. This difference was not observed for the proportion of peripheral blood Treg. No relation was found between the proportion of Treg and the amount of liver inflammation or liver damage. The liver contained a large population of CD4+CD25- cells that expressed FoxP3. These cells were only sporadically found in peripheral blood. Additionally, a higher proportion of liver Treg and liver CD4+ T cells expressed the immunoregulatory surface marker PD-1.

In conclusion, patients with a high viral load have a higher proportion of intra-hepatic Treg compared to patients with a low viral load which might be an explanation for the lack of control on viral replication.

Introduction

Worldwide 400 million people suffer from a chronic hepatitis B virus (HBV) infection and approximately 1 million people die annually from HBV-related disease. Infection with HBV in adults results frequently in a self-limiting, acute hepatitis, which confers protective immunity and causes no further disease. In 10% of infected adults HBV leads to a chronic infection. (1-3) In patients with an acute self limiting HBV infection, a multispecific $CD4^+$ and $CD8^+$ T cell response with a type 1 cytokine profile, is important for control of the infection. (4,5) Patients with a chronic HBV infection lack such a vigorous multispecific T cell response. (6,7) Patients with high levels of inflammation usually have large intra-hepatic infiltrates of non HBV-specific T cells, which are thought to be responsible for the destruction of hepatocytes. (6)

Regulatory T cells (Treg) play an important role in maintaining the peripheral immune tolerance. (8-10) Treg can be distinguished from CD4+ effector T cells by their continuous expression of the activation marker CD25 and the expression of the Treg specific transcription factor FoxP3. (11,12) Besides regulating peripheral tolerance, Treg can also affect T cell responses to foreign antigens. (13) Patients with a chronic HBV infection have increased percentages of Treg in their peripheral blood compared to healthy controls and individuals who have resolved their HBV infection. (14,15) Reducing the viral load by adefovir therapy resulted in a decrease in peripheral blood Treg. (16) Treg from patients with a chronic HBV infection are capable of inhibiting the HBV specific CD4+ and CD8+ T cell response in a dose dependent manner. (14,17,18) These studies however are predominantly focused on peripheral blood Treg. Since the site of infection for HBV is the liver, the aim of this study is to investigate the role of intra-hepatic Treg in chronic HBV infection.

Materials and methods

Patients. Forty patients underwent percutaneous needle liver biopsy as part of their diagnostic evaluation (table 1). All patients had detectable HBVDNA levels in serum. Patients co-infected with either human immunodeficiency virus, hepatitis A virus, hepatitis C virus or hepatitis D virus and patients with a resolved viral hepatitis were excluded from this study. Patients who were immunocompromised were

also excluded from participation in this study. Excess tissue from the liver-biopsy samples (not needed for histological examination) was used to isolate intra-hepatic lymphocytes. A venous blood sample was collected from each patient on the day of the biopsy. The institutional review board of the Erasmus MC – University Medical Center Rotterdam, approved this study, and informed consent was obtained from all patients prior to their inclusion in the study.

Table. Patient characteristics

Sex	male: 34 female: 6
Race	Caucasian: 19 Asian: 11 Negro: 10
HBV DNA (geq/ml)*	2.83×10^6 (1.00×10^3 - 1.90×10^{10})
ALT (U/L)*	55 (24-630)
HBeAg	21 HBeAg Positive 19 HBeAg Negative
Fibrosis	Metavir score 0: 8 1: 10 2: 14 3: 5 4: 3
Treatment	untreated: 29 adefovir: 11

* median (range)

Virological assessment. Serum HBeAg and anti-HBe were determined quantitatively using the Abbott IMX system (Abbot Laboratories, North Chicago, IL) according to the manufacturer's instructions. Serum HBV DNA was determined using an in-house developed TaqMan Polymerase chain reaction based on the Eurohep standard (detection limit, 373 geq/ml) (Applied Biosystems, Foster City, CA). (19)

Flowcytometry. PBMC were obtained by ficoll separation (Ficoll-Paque^{TM} plus, Amersham Biosciences, Buckinghamshire, UK). The liver tissue was collected in RPMI 1640 (Bio Whittaker, Verviers, Belgium) and was digested with 0.04% collagenase P (Roche, Mannheim, Germany) for 15 minutes at 37°C. After digestion

the cells were grinded through a 70 μm nylon cell strainer (BD Falcon, Bedford, MA) to create a single cell suspension.

The FoxP3 antibody staining was performed according to the manufacturer's instructions. Briefly, cell surface markers were stained with anti-CD25-PE (M-A251) (BD Pharmingen, San Jose, CA) and anti-CD4-PerCP-Cy5.5 (SK3) (Becton Dickinson, San Jose, CA). The cells were fixed and permeabilized with Fix/perm buffer and permeabilization buffer (eBiosciences, San Diego, Ca). Anti-FoxP3-APC (PCH101) (eBiosciences) was added during permeabilization. For three patients Anti-PD-1-FITC (MIH4) (BD Pharmingen) was added during the cell surface marker staining. For anti-CD25-PE, anti-PD-1-FITC and anti-FoxP3-APC isotype matched control antibodies were used. After staining the cells were analyzed using a four-color cytometer (FACScalibur™, CELL Quest Pro™ software, Beckton Dickinson).

Immuno-histochemistry. CD8+ and FoxP3 + cells in liver tissue slides were examined by immuno-histochemical staining with either anti CD8 (C8/14415) (Dako, Glostrup, Denmark) or anti-FoxP3 (236A/E7) (Abcam, Cambridge, United Kingdom). The liver sections were first deparaffinized with xylene (LabScan Ltd, Dublin, Ireland) and ethanol (Merck, Darmstadt, Germany). The antigen retrieval was performed by temperature-controlled incubation at 99°C in citrate buffer (pH 6.0) in a microwave followed by incubation with on of the antibodies. After incubation the liver specimens were washed and incubated with rabbit anti mouse immunoglobulins (RAM, Dako), followed by alkaline-phosphatase-anti-alkaline-phosphatase complex (APAAP, Serotec, United Kingdom). Fast Blue salt / naphtol AS-BI phosphate solution supplemented with levamisole (all from Sigma-Aldrich Chemie, Steinhem, Germany) was added to visualize the CD8+ or FoxP3+ cells. Nuclear Fast Red was used for counterstaining. Negative controls were performed by replacement of the primary antibody by an isotype-matched control antibody.

Statistical analysis. Data from the different patient groups was compared using a Mann Whitney U test. Flowcytometry data from PBMC and liver cells were compared using the Wilcoxon matched pairs signed rank sum test. For these analyses SPSS 11.5 for Windows (SPSS, Chicago, IL) was used.

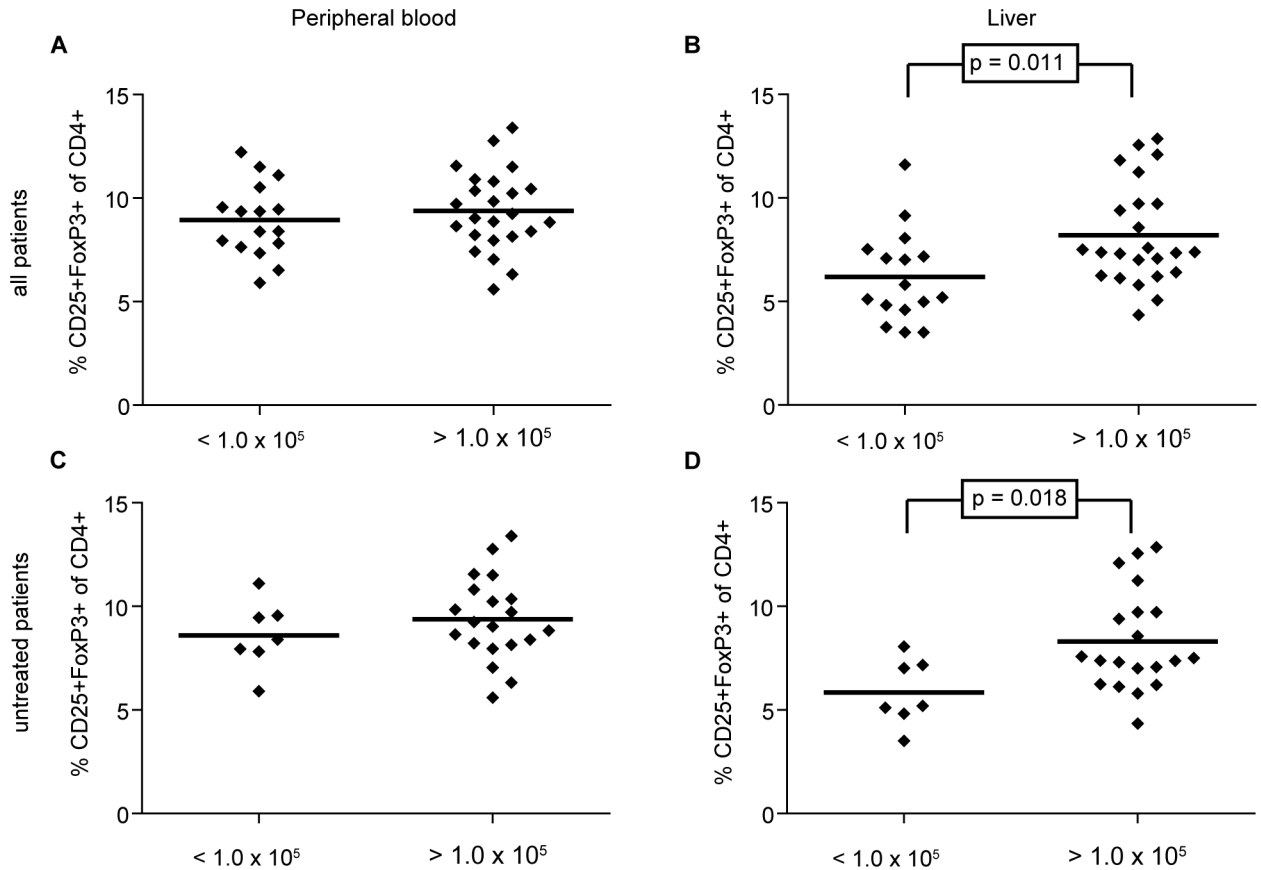

Figure 1. The proportion of Treg determined by flowcytometry is defined as the percentage of cells staining positive for CD4, CD25 and FoxP3 divided by the percentage of cells staining positive for CD4. The bar represents the mean proportion of Treg. Patients were divided into two groups (Viral load < 10^5 geq/ml and viral load > 10^5 geq/ml ,depicted on the x- axis). **(A)** Peripheral blood Treg of all patients (n=40). **(B)** Liver Treg of all patients (n=40). **(C)** Peripheral blood Treg of the untreated patients (n=26). **(D)** Liver Treg of the untreated patients (n=26).

Results

Patients with a high viral load have an increased proportion of intra-hepatic Treg. Forty patients underwent percutaneous needle liver biopsy as part of their diagnostic evaluation (table 1). All patients had detectable HBVDNA levels in serum. To determine whether there is an association between the viral load and the presence of intra-hepatic Treg, we collected small pieces of liver biopsy material (3 mm – 5 mm) and 10 ml of peripheral blood of 40 patients with a chronic HBV infection. We performed flowcytometry with antibodies to CD4, CD25, and FoxP3 on the liver derived mononuclear cells (LMNC) and PBMC. The proportion of Treg was defined as the proportion of cells that stained positive for CD25 and FoxP3 within the population of CD4 positive cells. When patients were divided into two groups, one group with a viral load of $<1 \times 10^5$ (geq/ml; n=16) and one group with viral load $>1 \times 10^5$ (geq/ml; n=24). No difference was observed in the proportion of peripheral blood Treg between patients with high viral load and a low viral load (8.94% ± 0.43% vs. 9.38% ± 0.39% respectively; figure 1A). In contrast, patients with a high viral load did have a higher proportion of intra-hepatic Treg compared to patients with a low viral load (6.18% ± 0.54% vs. 8.20 % ± 0.50% respectively, p= 0.011; figure 1B). To exclude the possibility that antiviral treatment affected our results, we performed the same analysis including only the proportion of Treg in untreated patients (n=26). For these patients the difference in the proportion of Intra-hepatic Treg between patients with a low viral load and patients with a high viral load was also observed (5.84% ± 0.65 % vs. 8.30% ± 0.54%, p = 0.018) and there was no difference observed in the proportion of peripheral blood Treg (8.60% ± 0.62 vs. 9.38% ± 0.45%; figure 1C and 1D).

There is no relation between liver damage and the proportion of Treg. Next we determined whether there was a correlation between the proportion of Treg and liver inflammation. No correlation between serum ALT levels and the proportion of peripheral blood Treg or intra-hepatic Treg was observed. When patients were divided into two groups based on their serum ALT levels (ALT < 2 x ULN; n=25 and ALT > 2 x ULN; n=15) no difference was observed between the two patient groups for peripheral blood Treg (9.34% ± 0.31% vs. 8.89 ± 0.60% respectively) as well as for intra-hepatic Treg (7.61% ± 0.56% vs. 7.03% ± 0.52% respectively) To asses

whether there was a relation between the amount of previous liver damage and the proportion of intra-hepatic Treg, the patients were divided into five groups based on their Metavir fibrosis score. (20) No difference was observed between the different groups for either the proportion of peripheral blood Treg or the proportion of intra-hepatic Treg (figure 2A and B). There were also no differences observed for the untreated patients alone (data not shown)

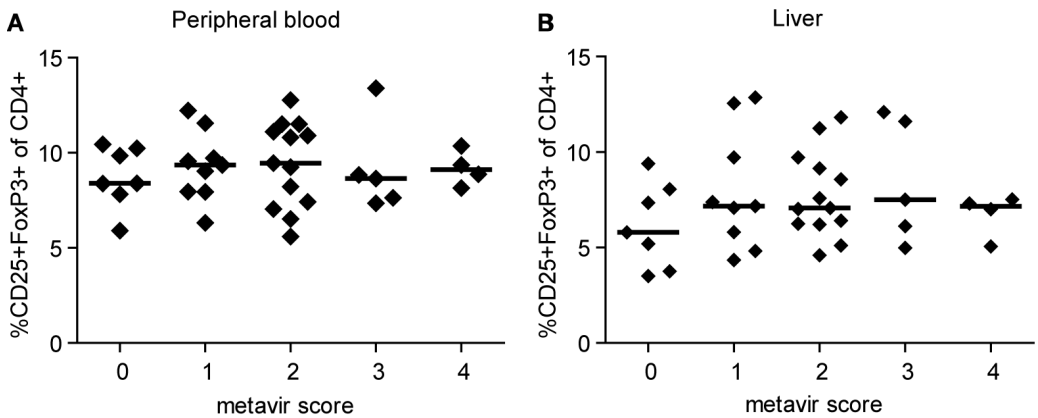

Figure 2. The proportion of Treg determined by flowcytometry is defined as the percentage of cells staining positive for CD4, CD25 and FoxP3 divided by the percentage of cells staining positive for CD4. The bar represents the mean proportion of Treg. Patients were divided into five groups based on their Metavir fibrosis score. **(A)** Peripheral blood Treg of all patients (n=40). **(B)** Liver Treg of all patients (n=40).

The liver contains a larger population of CD4+CD25-FoxP3+ cells. Treg are usually defined as CD4+CD25+FoxP3+ cells. However, in the liver we also detected a substantial population of CD4+CD25-FoxP3+ cells. A significantly lower frequency of CD4+CD25+FoxP3+ cells was detected in the liver as compared to peripheral blood (7.39% ± 0.40% vs. 9.21% ± 0.29% ($p < 0.001$) respectively; figure 3), whereas no difference was observed in the proportion of FoxP3+ cells of CD4+ cells (9.72% ± 0.49% vs. 10.10% ± 0.30% respectively; Figure 3).

Approximately 40% of liver Treg expressed the immunoregulatory marker PD-1 while peripheral blood Treg hardly expressed PD-1. However this is not specific for Treg, since the liver contains more PD-1+CD4+ cells as compared to peripheral blood. A representative staining for PD-1 on PBMC and LMNC is depicted in Figure 4.

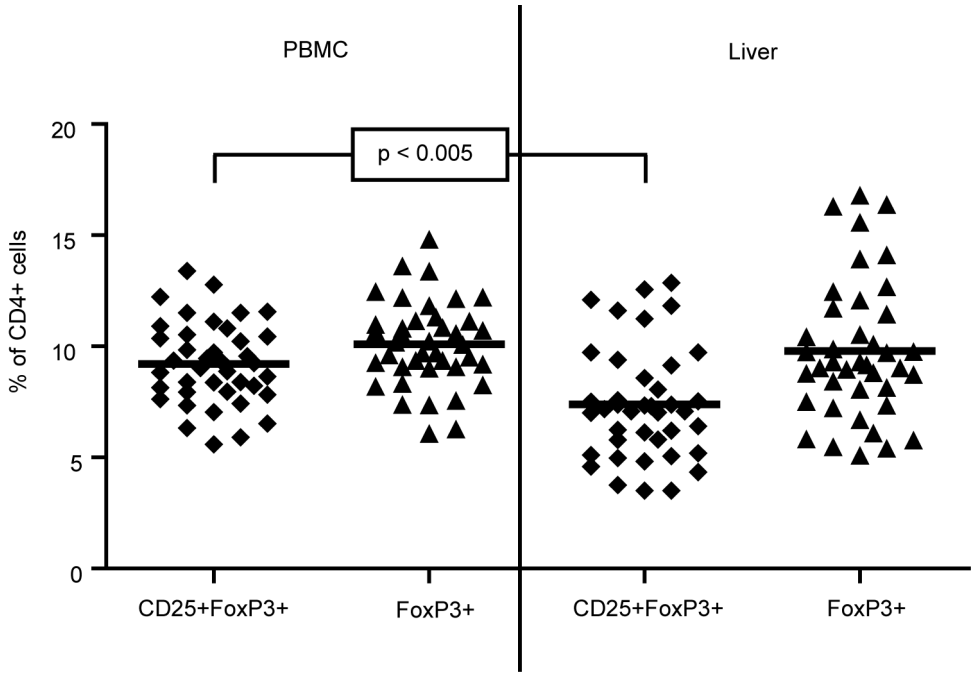

Figure 3. The proportion of CD25+FoxP3+ and CD25-FoxP3+ cells of CD4+ cells determined by flowcytometry in peripheral blood (left) and liver (right) (n=40).

FoxP3 positive cells are predominantly found in the portal infiltrate. To study the localization of the Treg in the liver tissue immuno-histochemistry for FoxP3 was performed on paraffin embedded liver tissue of five 5 patients. Immuno-histochemistry for CD8 was performed as a positive control. The CD8+ T cells were detected in the liver parenchyma (Figure 5A) as well as in the portal infiltrates (Figure 5B). FoxP3 positive cells however were predominantly seen in the portal infiltrates and hardly in the liver parenchyma (Figure 5C).

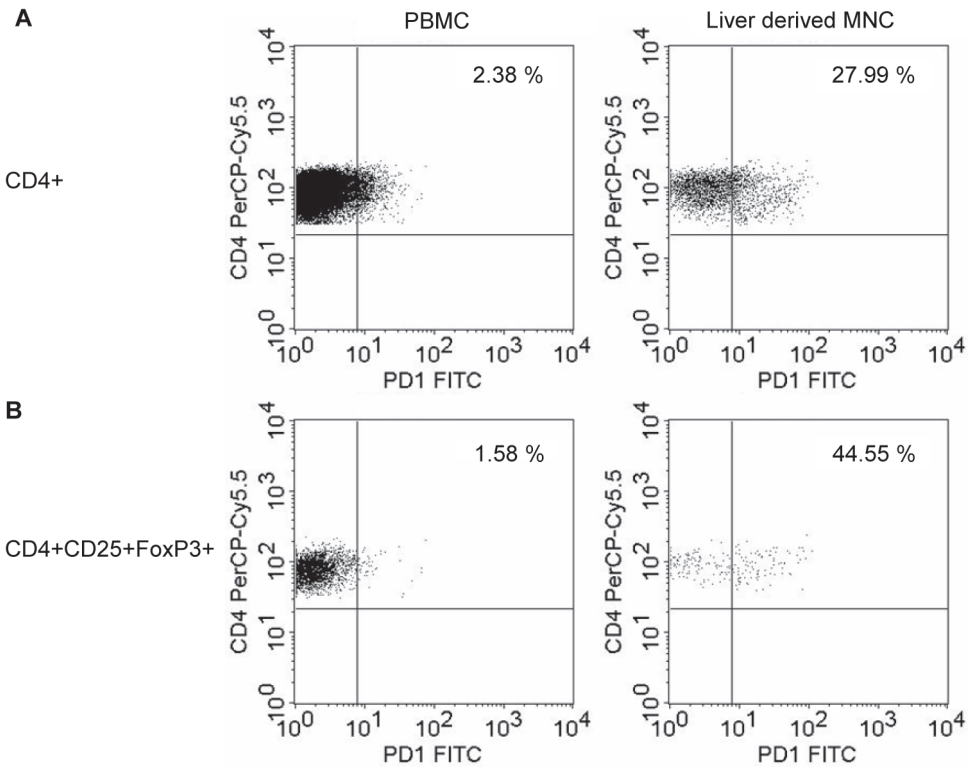

Figure 4. Representative staining for PD-1 (one out of three patients). PBMC are depicted on the left and LMNC are depicted on the right. **(A)** Dotplots of CD4+ cells. **(B)** Dotplots of the CD4+CD25+FoxP3+ cells (Treg). Gates were set using isotype matched control antibodies.

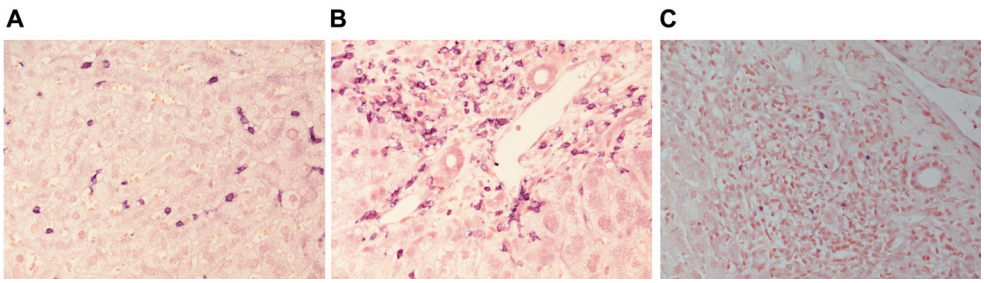

Figure 5. Nuclear fast red counterstained liver tissue of chronic HBV patients. **(A and B)** Staining with anti-CD8 and RAM visualized with Fast Blue substrate. **(C)** Staining with anti-FoxP3 and RAM visualized with Fast Blue substrate. Magnification 400x.

Discussion

The liver is an immunotolerant organ. (21) Kupffer cells and liver sinusoidal endothelial cells continuously express the immunoregulatory cytokines IL-10 and TGF-β. (22) TGF-β has been shown to be important for the induction of Treg. (23-26) The local cytokine environment of the liver therefore appears to be an ideal environment for Treg induction. Treg induction in the liver draining lymph node has also been described. (27) Furthermore, several studies have demonstrated increased numbers of peripheral blood Treg in patients with a chronic HBV infection. (14,15,18) These Treg are capable of inhibiting HBV-specific proliferation and IFN-γ production of HBcAg-specific CD4+ and CD8+ T cells. (14,17, 8)

When patients were divided into two groups (HBV DNA $<10^5$ and HBV DNA $> 10^5$), patients with a higher viral load had a higher proportion of intra-hepatic Treg. Treg can be induced through repetitive stimulation of T cells by the presence of high concentrations of antigen for longer periods of time. (28) This could be an explanation for the higher proportion of Treg observed in the chronic HBV patients with a high viral load. The increased proportion of intra-hepatic Treg in patients with a high viral load is not observed for the proportion of peripheral blood Treg. The difference in the proportion of intra-hepatic Treg was not affected by (anti-viral) therapy, since the same trend was observed after exclusion of all treated patients.

Treg play an important role in maintaining immune tolerance. (8-10) Patients with a high ALT often have massive liver inflammation and cell death and as a consequence a high level of self-antigen will be present. Therefore we expected to find a relation between the serum ALT levels and the proportion of intra-hepatic Treg. However this correlation was neither found for the proportion peripheral blood Treg nor for the proportion of intra-hepatic Treg. This is in contrast to what Xu *et al* have shown for peripheral blood Treg. (18) Also no relation between liver fibrosis and the proportion of intra-hepatic or peripheral blood Treg was observed. This indicates that the increased proportion of Treg observed in patients with a chronic HBV infection is not a result of liver damage and the presence of self-antigen caused by liver damage.

In this study we have shown that the liver contains a larger population of CD4+CD25-FoxP3+ cells compared to peripheral blood. It has been suggested that Treg can down regulate CD25 depending on their activation status. (29) However,

a mice study by Nishioka *et al* revealed that CD4+CD25-FoxP3+ cells are also capable of inhibiting the T cell response. (30) Since Treg have to be activated to become suppressiv, (31) this would suggest that the population of CD4+CD25-FoxP3+ cells in the liver is not a population of less activated Treg.

Next to the relatively large population of CD4+CD25-FoxP3+ cells we also detected increased expression of PD-1 on intra-hepatic Treg compared to peripheral blood Treg. The observed higher intra-hepatic PD-1 expression is not specific for Treg, since a higher proportion of the entire population of CD4+ cells in the liver express PD-1. It has recently been shown that PD-1 is involved in the suppressive mechanism of Treg during an allo-immune response and that Treg up regulate PD-1 mRNA after activation. (32) The increased expression of PD-1 on intra-hepatic Treg provides additional evidence that these Treg are not less activated compared to peripheral blood Treg.

Immuno-histochemistry showed that FoxP3 positive cells were predominantly localized in the portal infiltrate and CD8+ T cells were also found outside the portal infiltrates. In a previous study by our group, co-localization of CD8+ and infected hepatocytes was shown. (33) Although we did not perform double staining for FoxP3 and HBV core antigen or HBV surface antigen in this study, co-localization of Treg and infected hepatocytes seems very unlikely, since there were almost no FoxP3+ cells observed outside of the portal infiltrates. Since Treg are capable of inhibiting CD8+ T cell function the Treg will probably exert their suppressive effect on CD8+ T cells before the cytotoxic T cells enter the liver parenchyma. (17)

In conclusion, patients with a high viral load have a higher proportion of intra-hepatic Treg compared to patients with a low viral load which might be an explanation for the lack of control on viral replication.

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Inhibition of viral replication reduces regulatory T cells and enhances the antiviral immune response in chronic hepatitis B

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Abstract

Regulatory T cells (Treg) play a key role in the impaired immune response that is typical for a chronic Hepatitis B virus (HBV) infection. To gain more insight in the mechanism that is responsible for this impaired immune response, the effect of viral load reduction resulting from treatment with the nucleotide analogue adefovir dipivoxil, on the percentages of Treg and HBV specific T cell responses was analyzed. Peripheral blood mononuclear cells (PBMC) of 12 patients were collected at baseline and during treatment. In parallel to the decline in viral load, we found a decline in circulating Treg, combined with an increase in HBV core antigen-specific IFN-γ production and proliferation. The production of IL10 did not decrease during therapy.

In conclusion, adefovir induced viral load reduction results in a decline of circulating Treg together with a partial recovery of the immune response.

Introduction

Hepatitis B virus (HBV) is a common non-cytopathic DNA virus. Infection with HBV in adults usually results in a self-limiting, acute hepatitis, which confers protective immunity and causes no further disease. In 10% of infected adults HBV leads to a chronic infection. Chronic HBV infection is an important risk factor for the development of liver cirrhosis and hepatocellular carcinoma. Worldwide 400 million people suffer from a chronic HBV infection and approximately 1 million people die annually from HBV-related disease (1,2).

In patients with an acute self-limiting HBV infection, a multispecific CD4+ and CD8+ T cell response with interferon-γ (IFN-γ) production, is important for control of the infection (3,4). Patients with a chronic HBV infection lack such a vigorous multispecific T cell response, but instead exhibit a weak or undetectable virus-specific T cell response (3). Patients with a chronic HBV infection have increased percentages of CD4+CD25+ regulatory T cells (Treg) in their peripheral blood. Treg are capable of inhibiting the HBV specific immune response in a dose dependent manner and depletion of Treg results in an increased proliferation and IFN-γ production against HBV core antigen (HBcAg) (5). In addition, Xu *et al*. recently showed that patients with a high viral load have more peripheral Treg as compared to patients with a low viral load (6). It has been suggested that Treg can be induced through a repetitive stimulation of T cells by the presence of high concentrations of antigen for longer periods of time (7). The high viral load present in peripheral blood of HBV patients could possibly provide such a stimulus.

One of the therapeutic options for patients with a chronic HBV infection is antiviral treatment with nucleos(t)ide analogs (8). Adefovir dipivoxil is the prodrug of adefovir, an acyclic phosphonated adenine nucleotide analogue. Intracellularly, adefovir dipivoxil undergoes two phosphorylation steps to compete with deoxyadenosine triphosphate to inhibit viral DNA polymerase and HBV reverse transcriptase, resulting in chain termination of DNA synthesis (9).

The aim of this study was to determine how the decrease in viral load and antigenic pressure affects the percentage of Treg and the antiviral immune response, as tested by T cell proliferation, IFN-γ and IL10 production.

Materials and methods

Patients. Peripheral blood mononuclear cells (PBMC) from 12 chronic hepatitis B patients (10 males and 2 females, age 28 to 67 years; table 1) were obtained at baseline and after three and six months of adefovir treatment (10 mg orally per day). At baseline the median serum HBV DNA level was 1.7 X 10^8 geq/ml (range 2.0 X 10^4 - 2.1 X 10^9) and median alanine transaminase level (ALT), which is an indication for the extent of hepatocellular injury, was 119 U/l (range 43 - 467). Six patients were HBV e antigen (HBeAg) positive at baseline and all patients had biopsy proven chronic hepatitis with various degree of liver inflammation and fibrosis. Seven patients were treatment naive, and five patients had previously been treated with IFN-α and lamivudine (n=3) or with entecavir (n=2). Patients co-infected with Human Immunodeficiency virus, Hepatitis A virus, Hepatitis C virus (HCV) or Hepatitis D Virus and patients with other types of hepatitis were excluded from this study. Also patients who were pregnant or had received antiviral, immune suppressive or immunomodulatory treatment during the last six months were excluded from this study. All participants gave informed consent before blood sampling.

Tabel 1

Patient characteristics at baseline (n=12)	
Sex	10 male (83%)
Age	48 (28-67)* years
HBV DNA (copies/ml)	1.69 x 10^8 (2.00 x 10^4-2.14 x 10^9)*
Race	Asian: 5 (42%) Caucasion: 7 (58%)
ALT (Units/L)	119 (43-467)*
HBeAg positive	6 (50%)
Cirrhosis	2 (17%)
Previous treatment	treatment naive: 7 (58%) PEG Interferon + lamivudine: 3 (25%) Entecavir: 2 (17%)

* Median (range)

Virological assessment. Serum HBeAg and anti-HBe were determined quantitatively using the Abbott IMX system (Abbot Laboratories, North Chicago, IL) according to the manufacturers instructions. Serum HBV DNA was determined using

an in-house developed TaqMan Polymerase chain reaction based on the Eurohep standard (detection limit, 373 geq/ml) (Applied Biosystems, Foster City, CA)(10).

Isolation of the PBMC and Flow cytometric Analysis. PBMC were obtained by ficoll separation (Ficoll-Paque^{TM} plus, Amersham Biosciences, Buckinghamshire, UK). They were immediately frozen in RPMI 1640 (Bio Whittaker, Verviers, Belgium) containing 20% fetal calf serum (Hyclone, Logan, UT) and 10% dimethyl sulfoxide and stored at –135°C. PBMC from different time points were tested simultaneously to avoid interassay variations in individual patients. Flow cytometric analysis was performed on the stored samples using fluorochrome conjugated antibodies specific for the surface markers CD4, CD45RO and CD25 diluted in PBS/ 0.3% bovine serum albumin. The cells were fixed by incubation with intraprep reagent 1 and permeabilized by incubation with intraprep reagent 2 (Beckman-Coulter, Marseille, France). Anti-CTLA-4 antibody was added during permeabilization. The following antibodies were used: anti-CD4-PerCP-Cy5.5 (SK3) (Pharmingen, San Diego, CA), anti-CD45RO-APC (UCHL1) (Becton Dickinson, San Jose, CA), anti-CD25-FITC (2A3) (Becton Dickinson), anti-CTLA-4-PE (BNI3) (Immunotech, Marseille, France). For the anti-CD45RO-APC, anti-CD25-FITC, and CTLA-4-PE, isotype matched control antibodies were used to determine the level of background staining. The FoxP3 antibody staining was performed according the manufacturers instructions. Briefly, cells surface markers were stained with anti-CD25-PE (M-A251) (Pharmingen) anti-CD4-PerCP-Cy5.5 (SK3) (Pharmingen). The cells were fixed and permeabilized with Fix/perm buffer and permeabilization buffer (eBiosciences, San Diego, Ca) anti-FoxP3-APC (PCH101) (eBiosciences) was added during permeabilization. For anti-CD25-PE and anti-FoxP3-APC isotype matched control antibodies were used. After staining the cells were analyzed using a four-color cytometer (FACScalibur^{TM}, CELLQuest Pro^{TM} software, Beckton Dickinson).

Proliferation assay. The proliferation of mainly CD4+ T cells was determines as described earlier (5). Briefly, PBMC were cultured in triplicate in a concentration of $1x10^5$ cells per well in 100 µl RPMI 1640 (Bio Whittaker) containing 5% pooled human serum (Dept. of Immunohematology and Bloodbank, Leiden University Medical Center, Leiden, The Netherlands) and penicillin/streptomycin (Gibco, Paisley, UK). The cells were stimulated with 1 µg/ml HBcAg (a kind gift by M. van Roosmalen, Biomerieux, Boxtel, The Netherlands), 15 limits of flocculation/ml purified tetanus

toxin (SVM, Bilthoven, The Netherlands) or not stimulated and cultured for six days. After five days of incubation the cells were pulsed with 0.25 µCi/well of [^{3}H]-thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK). The cells were harvested 16 hours later. Proliferation was determined by liquid scintillation counting of the harvested cells.

IFN-γ and IL10 Elispot. The IFN-γ production of CD4+ T cells was determined by Elispot as described earlier (11). Briefly, PBMC were cultured in triplicate in a concentration of 1x10^5 cells per well in 100 µl RPMI 1640 (Bio Whittaker) containing 5% pooled human serum (Dept. of Immunohematology and Bloodbank, Leiden University Medical Center) and penicillin/streptomycin (Gibco). The cells were stimulated with either 1 µg/ml HBcAg, 20 µg/ml Phytohemagglutinin (PHA) (Murex) or 15 limits of flocculation/ml purified tetanus toxin (SVM) or were left unstimulated and cultured for 24 hours. After 24 hours the PBMC were transferred to antibody coated Elispot immunoplates. The IFN-γ Elispot kit was obtained from U-CyTech (Utrecht, The Netherlands) and the IL10 Elispot kit was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Both assays were performed according to the manufacturer's instructions. The spots were counted using a Bioreader 3000 from BioSys (Karben, Germany). The number of spots depicted represents the number of spots produced by stimulated cells minus the number of spots produced by unstimulated cells.

In vitro effect of adefovir on the functioning of PBMC. 9-(-2)-phosphonylmethoxy-ethyladenine (PMEA) is the active metabolite of adefovir (12). PMEA was kindly provided by Gilead Sciences Inc. (Foster City, CA). PBMC from untreated HBV patients were stimulated with HBcAg in the presence of 100 ng/ml, 10 ng/ml, 1 ng/ml and 0.1 ng/ml of PMEA or no PMEA. Unstimulated PBMC and unstimulated PBMC in the presence of 100 ng/ml of PMEA were used as a negative control. The proliferation and IL10 production were determined by [^{3}H]-thymidine incorporation and Elispot for all conditions.

Statistical Analysis. All flow cytometry and functional data were compared to levels at baseline using a Wilcoxon matched pairs signed rank sum test. SPSS version 11.5 for Windows (SPSS, Chicago, IL) was used. Correlations were determined using Spearman's correlation test. Results are given in mean ± SEM, unless specified otherwise.

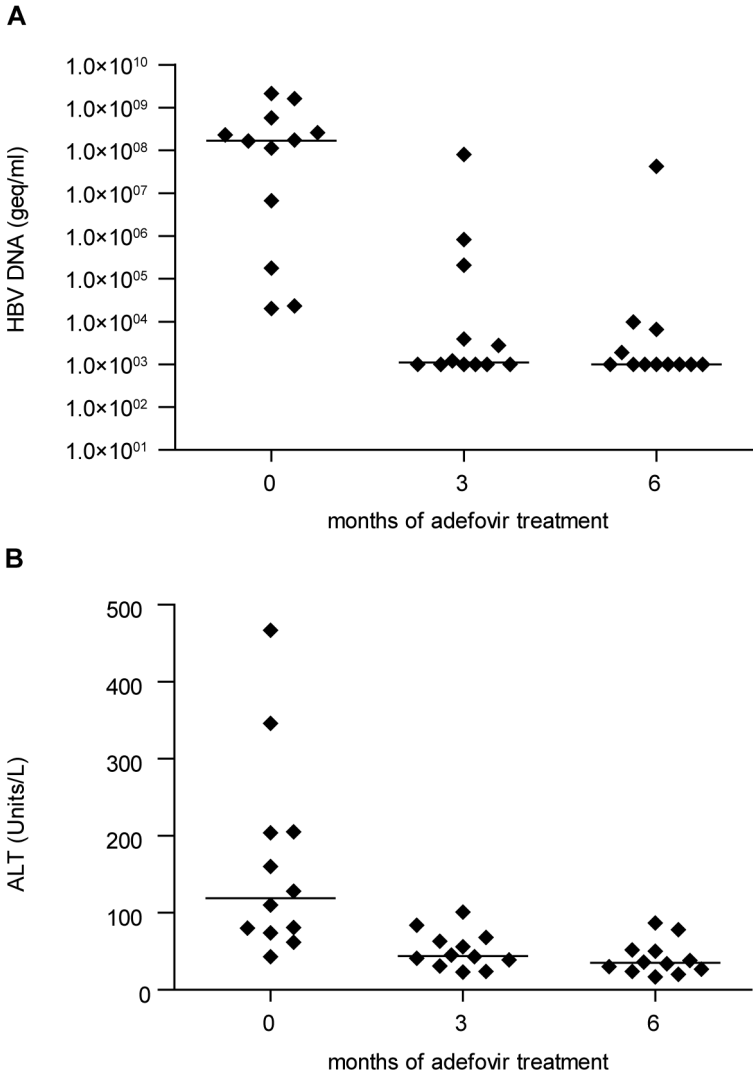


Figure 1. A) The serum HBV DNA levels of the 12 patients at baseline after three months and after six months of adefovir therapy. The bar represents the median serum HBVDNA level. **B)** The serum ALT levels of the 12 patients at baseline after three months and after six months of adefovir therapy. The bar represents the median serum ALT level. HBV DNA and ALT levels were significantly lower at three and six months compared to baseline ($p < 0.05$).

Results

HBV DNA decreased and alanine aminotransferase levels normalized during adefovir treatment. Serum HBV DNA level and alanine aminotransferase

level (ALT) were determined at baseline, as well as after three and six months of treatment (figure 1). The median reduction in serum HBV DNA from baseline to month six was 5.2 $\log_{10.}$ Six months of treatment led to undetectable HBV DNA in nine (75%) patients and to normalization of the ALT levels in eight (66%) patients; Eight (66%) patients reached both endpoints. Three patients had seroconversion from HBeAg to anti HBeAg during treatment. No patient exhibited loss of serum HBsAg.

The proportion peripheral blood Treg decreased during adefovir induced viral load reduction.

To assess the effect of treatment with adefovir on the immune response we first determined the proportion of Treg from cells that stained positive for CD4. Treg were defined as the percentage of cells positive for CD4, CD25, CD45RO and CTLA-4 divided by the percentage CD4 positive cells (figure 2A). After three and six months of therapy we found a significant decrease in the percentage of Treg (baseline 4.0 ± 0.4 vs. three months 3.2 ± 0.3 vs. six months 3.3 ± 0.4, $p = 0.019$ and $p = 0.037$ respectively; figure 2B). A weak correlation was observed between the percentage decrease in HBVDNA and the percentage decrease in the proportion of Treg ($r = 0.384$ and $p < 0.05$; data not shown).

Since FoxP3 is considered to be the most specific marker for Treg, we also performed a flowcytometry staining with antibodies against CD4, CD25 and FoxP3 in four of our patients (figure 3A). For this the percentage of cells that stained positive for CD4, CD25 and FoxP3 was divided by the proportion of CD4 positive cells. All four patients had a decrease in the proportion of Treg (10% decrease ± 3.5%) during the first three months of treatment. After six months of treatment the proportion of Treg increased somewhat, but remained lower than at baseline. These results concurred with the CD4, CD25, CD45RO and CTLA-4 Treg staining for all four patients (figure 3B). To determine whether the CD4+CD25+CTLA-4+ cells are FoxP3+ Treg, flowcytometry was performed with those four markers on PBMC from healthy controls. FoxP3 was expressed by 97% of the CD4+CD25+CTLA-4+ cells. One representative healthy control is depicted in figure 3C.

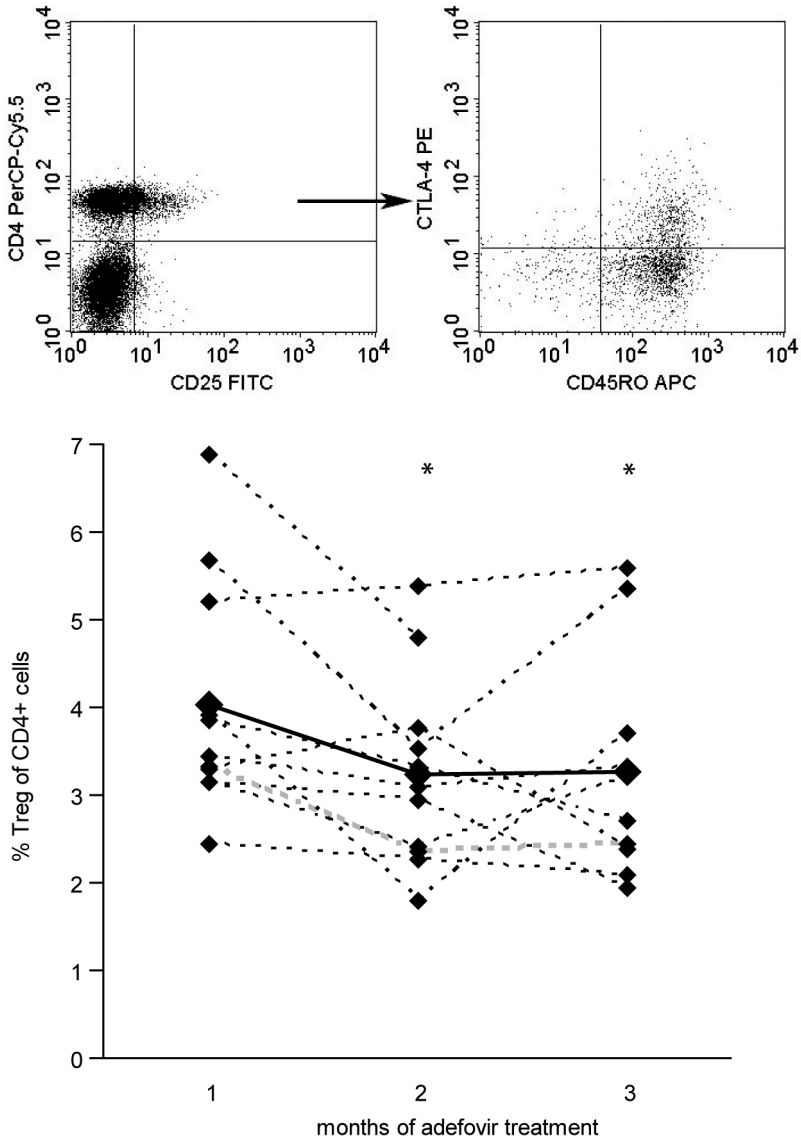


Figure 2. A) Flow cytometry staining experiment from a representative patient. The Treg staining was performed using antibodies against CD4, CD25, CD45RO and CTLA-4. The cells depicted in the upper right quadrant of the left dot plot are CD4+CD25+ and are depicted in the right dot plot. The cells in the upper right quadrant of the right dot plot are CD4+CD25+CD45RO+CTLA-4+ and are considered to be Treg. The gates were set using isotype matched control antibodies. **B)** The percentage of Treg defined as the percentage of cells that stained positive for CD4, CD25, CD45RO and CTLA-4 divided by the percentage of cells that stained positive for CD4. The dotted lines represent the individual patients and the solid line depicts the mean percentage Treg.* $p < 0.05$ compared to baseline.

Proliferation and IFN-γ production increased during adefovir induced viral load reduction. To determine the (HBV) specific proliferation and IFN-γ production, PBMC collected at baseline and during therapy were stimulated with HBcAg or tetanus toxin. As compared to baseline the HBcAg specific proliferation had increased both after three and six months of adefovir treatment (baseline 2893 CPM ± 689 vs. three months 5372 CPM ± 1382 vs. six months 4287 CPM ± 906; figure 4A). A similar increase was found for the proliferation to Tetanus toxin (figure 4B). The percentage decrease in HBVDNA and the percentage increase in proliferation to HBcAg was weakly correlated ($r = 0.371$ and $p < 0.05$; data not shown).

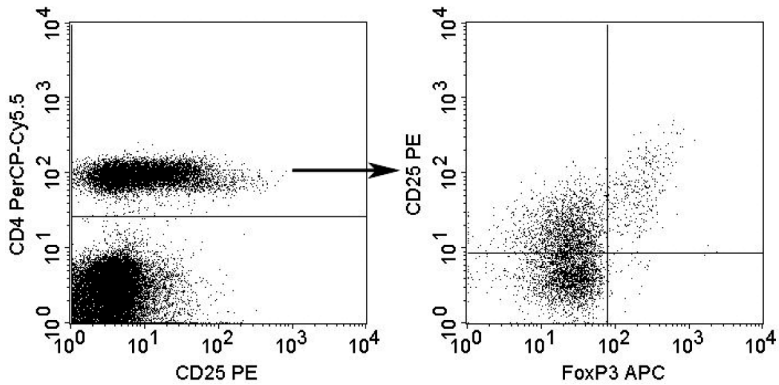
PBMC from healthy controls did not proliferate upon stimulation with HBcAg (data not shown).

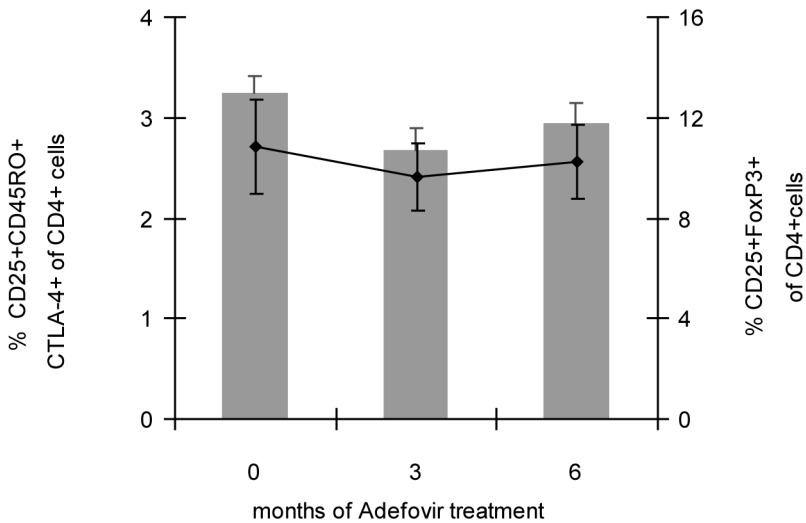
In parallel, the number of IFN-γ producing cells as determined by Elispot significantly increased during treatment both to HBcAg (5.7 spots/ 100,000 cells ± 1.8 at baseline vs. 17.5 spots 100,000 cells ± 4.9 at three months vs. 15.7 spots / 100,000 cells ± 5.0 at six months, $p = 0.014$ and $p = 0.014$ respectively; figure 4C) as well as to tetanus toxin (figure 4D).

Adefovir induced viral load reduction did not lead to a reduced IL10 production. Since clearance of HBV requires a cytokine response with a T helper 1 type profile, we determined the effect of adefovir therapy on the anti-inflammatory cytokine IL10. For this purpose PBMC collected at baseline and during therapy were stimulated with HBcAg or with PHA as a positive control. The number of IL10 producing cells was determined by Elispot. Treatment resulted in a non-significant increase (baseline vs. six months; $p = 0.155$) in the number of IL10 producing cells upon stimulation with HBcAg (baseline 24.9 spots/ 100,000 cells ± 6.6 vs. three months 28.7 spots 100,000 cells ± 8.11 vs. six months 37.6 spots / 100,000 cells ± 8.0; figure 5)

Adefovir had no direct effect on PBMC. The increased proliferation of PBMC upon stimulation with HBcAg during adefovir treatment could be caused by a direct effect of adefovir on PBMC or by decrease in viral load. PMEA is the active metabolite of adefovir. To examine whether PMEA has a direct stimulatory activity PBMC from untreated HBV patients were stimulated with HBcAg in culture medium supplemented with different concentrations of PMEA. This agent had no direct stimulatory effect resulting in proliferation (Figure 6A) or IL10 production (Figure

6B; n=6). When stimulated with HBcAg different concentrations of PMEA had also no effect on the proliferation of PBMC (Figure 6A) or the number of IL10 producing cells (Figure 6B).





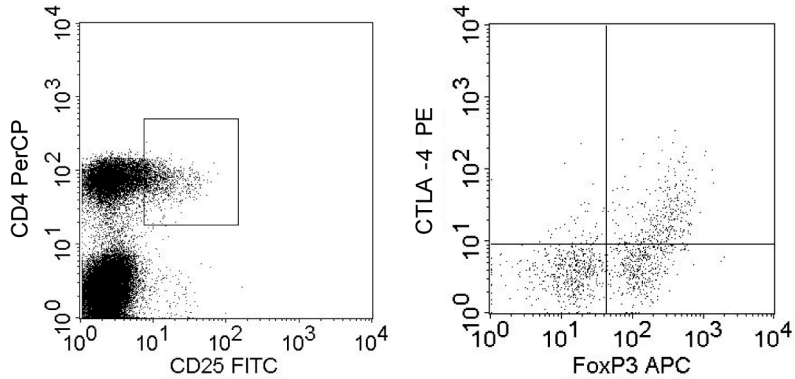


Figure 3. A) The Treg staining was performed with antibodies against CD4, CD25 and FoxP3. The cells depicted in the gate of the left dot plot are CD4+ and are depicted in the right dot plot. The cells in the upper right quadrant of the right dot plot are CD4+CD25+FoxP3+ and are considered to be Treg. The gates were set using isotype matched control antibodies. **B)** The percentage of Treg defined as the percentage of CD4+CD25+FoxP3+ cells divided by the percentage of CD4+cells (line and right y-axis) or the percentage of CD4+CD25+CD45RO+CTLA-4+ cells divided by the percentage of CD4+ cells (bars and left y-axis) (n=4). The change in percentage of Treg follows the same trend for both staining methods. Data are expressed as mean ± SEM. **C)** Flow cytometry staining experiment from a representative healthy control. The staining was performed using antibodies against CD4, CD25, CTLA-4 and FoxP3. The cells depicted in the gate of the left dot plot are CD4+CD25+ and are depicted in the right dot plot. The cells in the upper right quadrant of the right dot plot are CD4+CD25+CTLA-4+FoxP3+. The gates were set using isotype matched control antibodies.

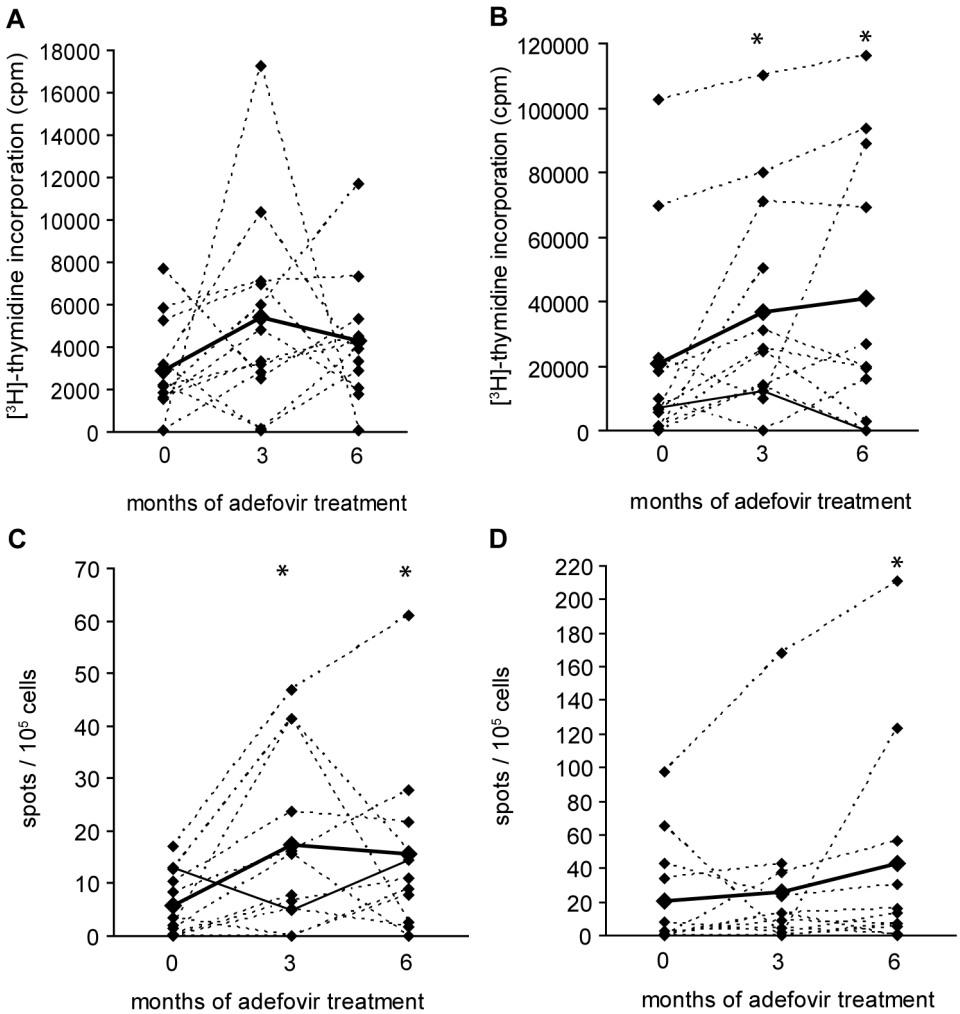


Figure 4. PBMC obtained at baseline, after three months and after six months of treatment were stimulated for six days with HBcAg **A)**. or Tetanus toxin. **B)**. The proliferation was determined by the incorporation of [^{3}H]-thymidine. PBMC were also stimulated for 48 hours with HBcAg **C)**. or Tetanus toxin **D)**. IFNγ production was determined by Elispot. The dotted lines represent the individual patients and the solid line depicts the mean, * $p < 0.05$ compared to baseline.

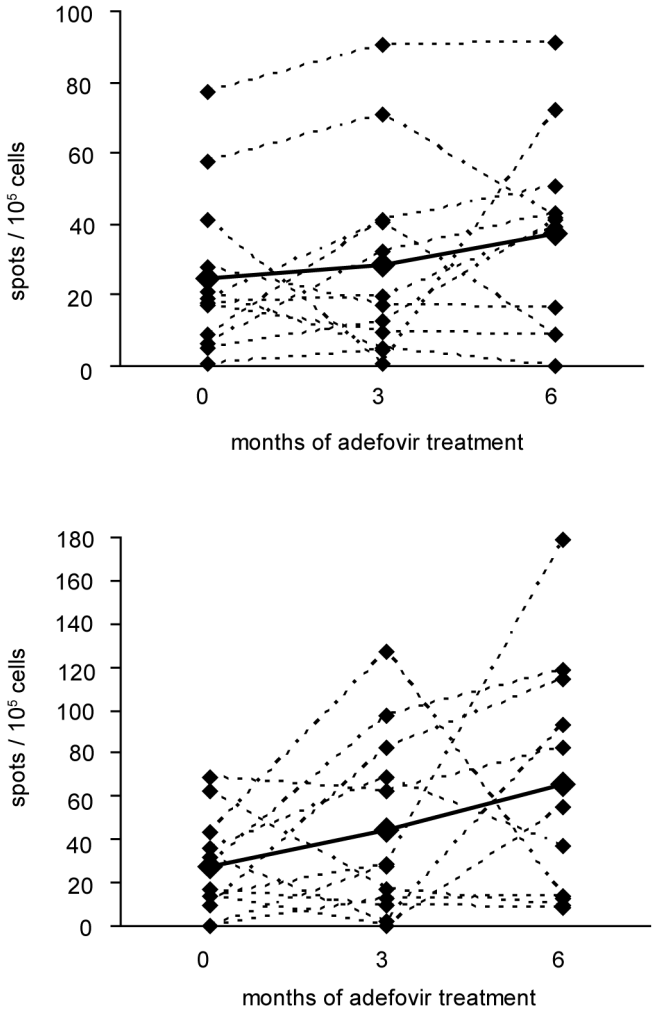


Figure 5. PBMC from baseline, after three months and after six months of were stimulated for 48 hours with HBcAg **(A)** or PHA **(B)**. The IL10 production was determined by Elispot. The dotted lines represent the individual patients and the solid line depicts the mean.

Discussion

Patients with chronic HBV infection lack the vigorous multispecific CD4+ and CD8+T cell response with a type 1 cytokine profile necessary to clear the infection

(3, 13). The precise mechanism responsible for this impaired T cell response is not known. Treg represent a unique lineage of T cells with a critical role in maintaining immune homeostasis. Treg are very potent suppressors of the immune response. It has recently been shown that a slight decrease in percentage of Treg (1,3%) can result in the development of autoimmune disease (14). Previously, we have shown that Treg contribute to the impaired immune response in patients with a chronic HBV infection by inhibiting the proliferation and IFN-γ production to HBcAg in a dose dependent manner (5). Treg are also capable of inhibiting the proliferation and IFN-γ production of HBV-specific CD8+ T cells (15).

In the current study we observed a decrease in the percentage of Treg after the first three months of adefovir treatment, the time-period in which the most prominent decrease in viral load occurs. Simultaneously we observed an increase in the proliferation and the number of IFN-γ producing cells upon stimulation with HBcAg. This increased response to HBcAg is most likely an increase in CD4+ T cell functioning, since HBcAg has been described to induce predominantly a CD4+ T cell response (11). After six months of adefovir treatment there was no further decline in Treg and no further increase in the number of IFN-γ producing cells and proliferation to HBcAg. Accordingly, there was no further decline in the viral load during this period. This indicates that the percentage of Treg in the peripheral blood inversely correlates to the viral load. Furthermore, we showed that PMEA, the working metabolite of adefovir, has no direct effect on the proliferation and cytokine production to HBcAg. In addition, in vitro treatment with PMEA also had no effect on the proportion of Treg in the PBMC fraction (data not shown). The increased response we observed is therefore not caused by a direct effect of adefovir on the HBcAg specific proliferation. Since, we have compared the proportion of Treg in peripheral blood and not the absolute number, the relative decrease in Treg could be caused by an increase in the number of responder T cells. However if this is the case, adefovir induced viral load reduction still leads to a more favorable balance between effector and suppressor (Treg) T cells in the peripheral blood.

It has been suggested that Treg can be induced through a repetitive stimulation of T cells by high concentrations of antigen for longer periods of time (7). Adefovir inhibits viral replication and antigen production, which could potentially result in a decrease of Treg induction, leading to restoration of the immune response. In a previous study it was shown that lamivudine-induced reduction of viral load resulted in an increased CD4+ and CD8+ T cell response (16,17). Most likely, this increased

immune reactivity on lamivudine may also have been the result of a decrease in the percentage of Treg.

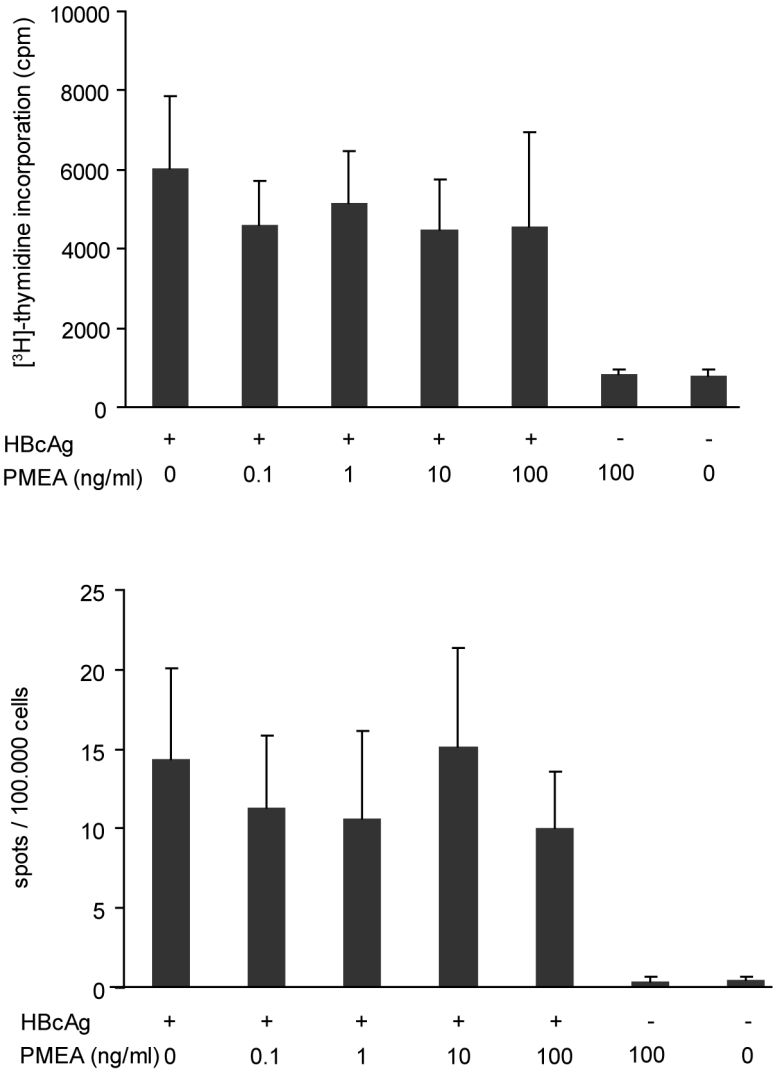


Figure 6. PBMC were stimulated for six days with HBcAg antigen (n=6) in the presence of different concentrations of PMEA. **A)** The proliferation was determined by the incorporation of [3H]-thymidine. **B)** PBMC were stimulated for 48 hours with HBcAg (n=6) in the presence of different concentrations of PMEA. The HBcAg specific IL10 production was determined by Elispot (n=6). Data are expressed as mean ± SEM.

In the current study and previously, we determined the percentage of Treg with antibodies against CD4, CD25, CTLA-4 and CD45RO (5). Recently, an antibody against FoxP3 has become available. The transcription factor FoxP3 is the most specific marker for Treg (18, 19). Therefore we also used a Treg staining with antibodies against CD4, CD25 and FoxP3. Both staining methods showed the same trend, however with the CD4+CD25+CD45RO+CTLA-4+ antibody staining we detected lower percentages of Treg compared to the FoxP3 antibody staining. Treg are CD4+ cells known to have high CD25 expression (20). In the current study all CD4+ $CD25^{high}$ cells expressed FoxP3 (figure 3A). Additional flowcytometry experiments revealed that 97% of the CD4+CD25+CTLA4+ cells expressed FoxP3, but not all FoxP3 positive cells expressed CTLA-4 (data not shown). Since CTLA-4 might have a functional role in the Treg-induced immune suppression, CTLA-4 positive Treg could well represent an activated sub-population of the FoxP3+ Treg (21-23).

The response to the control antigen (tetanus toxin) was also increased. This could be explained by a decrease in activated HBV-specific Treg. Treg have to be activated through their T cell receptor, but once activated the suppression by Treg is non-specific (23,24). Therefore, the decrease in viral load will result in less HBV-specific activated Treg capable of inhibiting the response of also non HBV-specific T cells.

The number of IL10 producing cells was not reduced during treatment. The lack in decrease of cells producing this immunoregulatory cytokine could be one of the reasons that antiviral therapy does not lead to a sustained antiviral response, but also suggests that Treg are not solely responsible for the hyporesponsiveness in chronic HBV patients. Recently, another regulatory T cell type, besides CD4+CD25+Treg, has been described. These so called T regulatory type 1 cells (Tr1) are CD4 positive cells and their suppression is mediated through an IL10 dependent mechanism (25). These Tr1 cells also play a dominant role in the latent immune response against Epstein Bar virus (26) and could be responsible for part of the IL10 production by PBMC cells observed in patients with a chronic HBV infection (27). Since the viral load reduction caused by adefovir is not sufficient to restore the antiviral immune response, as depicted by the unchanged production of the immuno regulatory cytokine IL10, combination therapy with antiviral drugs and immunomodulatory therapy may be more successful in perpetuating the antiviral immune response after treatment discontinuation

In conclusion, viral load reduction induced by treatment with adefovir results in a partial restoration of the impaired immune response, as indicated by a decrease in percentages of Treg as well as an increased HBV-specific proliferation and IFN-γ production. IL10 production, however, remained unaffected. The decrease in Treg is most likely established by the removal of the constant activation of the immune system by the high viral load.

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Induction of CD4+ CD25+ regulatory T-cells and IL-10 producing cells is associated with non-response to pegylated Interferon-α therapy for chronic hepatitis B virus infection

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Antiviral therapy, in press

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Abstract

Little is known about why treatment with interferon alpha (IFNα) leads to a response in only a minority of patients with chronic hepatitis B virus (HBV) infection. It was recently shown that in chronic HBV patients CD4+ CD25+ regulatory T-cells (Treg) can suppress the HBV-specific immune response. We aimed to investigate whether in non-responders to IFNα therapy Treg contribute to treatment failure by down-regulating the HBV-specific T-cell responses.

Fourteen HBeAg positive patients received pegylated (Peg) IFNα monotherapy for 52 weeks and were followed for 26 weeks.

Compared to non-responders, responders displayed an increased HBV-specific Th-cell proliferation. At the start of treatment there was no difference in the frequencies of CD4+ CD25+ Treg between responders and non-responders. During therapy, the frequency of CD4+ CD25+ Treg increased in non-responders but not in responders. In contrast to the responders, the non-responders showed a significant increase in frequency of IL-10 producing cells. Treg depletion resulted in increased proliferation capacity, but did not affect the frequency of IL-10 producing cells measured during the course of the treatment.

In conclusion, this study indicates that there may be an important role for Treg, in HBV-persistence during and after Peg-IFNα therapy.

Introduction

Chronic infection with hepatitis B virus (HBV) is characterized by inflammatory liver disease of variable severity and is a major cause of liver failure and hepatocellular cancer world-wide (1,2). HBV is a non-cytopathic virus, and liver injury is mainly mediated by the host immune response against virus infected liver cells (3). Therefore, in chronic hepatitis B infection (CHB) effective therapy is needed to stop viral replication and progression of liver damage.

Currently, interferon alpha (IFNα), a drug that modulates the anti-viral immune response, represents one of the most important treatment options in CHB (4-6). However, in only 20-30% of CHB patients conventional IFNα therapy leads to sustained virological response (7,8) and the introduction of pegylated-IFNα, a drug with a prolonged half-life, has not changed the response rate substantially (9,10).

Previous studies have defined two T helper cell subsets, Th1 and Th2, which are characterized by distinct and mutually exclusive patterns of cytokine production and different functions (11). Th1 cells produce IFNγ, IL-2 and promote cellular immune reactions, while Th2 cells produce IL-4, -5, -and IL-13, and enhance humoral immune response (11). Resolution of acute HBV infection has been associated with a vigorous polyclonal and multispecific Th1 response (12), whereas in the blood of chronically infected patients the HBV-specific Th1-response is weak, antigenically restricted or undetectable (13, 14). Peripheral T cells contain an immunoregulatory sub-population which expresses CD4, CD25 (the IL-2 receptor α-chain) and CD45RO, and have a continuous intracellular expression of the cytotoxic T lymphocyte-associated antigen-4 (CTLA-4). These regulatory T cells (Treg) express the forkhead/ winged helix transcription factor gene (*Foxp3*) (15), and they are capable of inhibiting the effector functions of T helper cells, cytotoxic T cells and natural killer T cells (16-19). In recent studies it was shown that patients with a chronic HBV infection have a higher percentage of Treg in their peripheral blood compared to healthy controls and individuals with a resolved HBV infection (20, 21). These Treg were able to suppress the HBV-specific T-cell response in a dose dependent manner.

Little is known about why treatment with pegylated IFN leads to a response in only a minority of patients. Elucidating the mechanism responsible for treatment failure may result in improved control of the virus infection and better treatment strategies. Therefore, in this study, we aimed to analyze longitudinally the virus-

specific T-cell responses and cytokine patterns during and after treatment with Pegylated IFNα-2b monotherapy. We hypothesized that in non-responders to IFNα therapy Treg contribute to treatment failure by down-regulating the HBV-specific Th1 responses.

Patients and methods

Patients. Patient material used for this study was derived from individuals that participated in a multicenter, randomized, double-blind study carried out to compare the efficacy of pegylated interferon-alpha 2b (Peg-IFN) monotherapy to a combination regimen with lamivudine for treatment of patients with HBeAg positive CHB (10). All patients were HBeAg positive, had alanine amino transferase (ALT) levels twice the upper limit of normal, had not received antiviral therapy within 6 months prior to enrollment and displayed no serious co morbidity. Patients were treated for 32 weeks with 100μg Peg-IFN weekly and with 50μg Peg-IFN weekly for another 20 weeks (total 52 weeks) after which they were followed-up for 26 weeks. Response to therapy was defined as HBeAg-loss at the end of follow-up. For the current study we used material from all patients enrolled in our institution that had received Peg-IFN monotherapy, and from whom material was obtained after signing informed consent (n=14; 8 responders, 6 non-responders) (table 1).

Isolation of the PBMCs and Flow Cytometric Analysis. At the start of therapy (t=0), 8 weeks later (t=8), at the end of therapy (t=52) and at the end of follow-up (t=78) PBMCs from patients were obtained by ficoll separation (Ficoll-Paque™ plus, Amersham Biosciences, Buckinghamshire, UK). The PBMCs were immediately frozen in medium containing 10% DMSO and stored at –135°C until further use. PBMC from different timepoints were tested simultaneously to avoid inter assay variations. Flow cytometric analysis was performed on the stored samples using fluorochrome conjugated antibodies specific for the surface markers CD4, CD45RO and CD25 diluted in PBS/ 0.3% bovine serum albumin. The cells were fixed by incubation with intraprep reagent 1 and permeabilized by incubation with intraprep reagent 2 (Beckman-Coulter, Marseille, France). Anti-CTLA-4 antibody was added during permeabilization. The following antibodies were used: anti-CD4-

PerCP-Cy5.5 (SK3) (Pharmingen, San Diego, CA), anti-CD45RO-APC (UCHL1) (Becton Dickinson, San Jose, CA), anti-CD25-FITC (2A3) (Becton Dickinson), anti-CTLA-4-PE (BNI3) (Immunotech, Marseille, France). For the CD45RO, CD25 and CTLA-4 antibodies, isotype matched control antibodies were used to determine the level of background staining. After staining the cells were analyzed using a four-color cytometer (FACScalibur^{TM}, CELLQuest Pro^{TM} software, Beckton Dickinson). The FoxP3 antibody (clone PCH101, eBiosciences, San Diego, Ca) staining was performed according to the manufacturers instructions. Briefly, cells surface markers were stained with anti CD25-PE (M-A251) (Pharmingen) anti-CD4-PerCP-Cy5.5 (SK3) (Pharmingen). The cells were fixed and permeabilized with Fix/perm buffer and permeabilization buffer (eBiosciences) anti-FoxP3-APC was added during permeabilization. As described previously (20), FoxP3 is expressed by CD25^{HI} CD4+ T cells and in FACS analysis of FoxP3-expression we gated on this cell population. To determine the level of background staining, for anti-CD25-PE and anti-FoxP3-APC isotype matched control antibodies were used.

Table I. Patient characteristics

Patients (case)	Sex	Age	Race	Baseline ALT (U/l)	Baseline HBV-DNA (geq/ml)	Response to therapy
1	Male	58	Cauc.	78	$1.6x10^9$	-
2	Male	47	Asian	125	$1.9x10^8$	-
3	Male	32	Asian	47	$1.6x10^8$	-
4	Male	27	Cauc.	70	$3.0x10^9$	-
5	Male	28	Cauc.	325	$7.1x10^9$	-
6	Male	49	Cauc.	394	$5.7x10^9$	-
7	Male	38	Cauc.	79	$7.9x10^9$	-
8	Female	21	Cauc.	160	$2.2x10^8$	-
9	Male	35	Cauc.	151	$5.2x10^9$	+
10	Male	41	Asian	131	$1.2x10^9$	+
11	Male	43	Cauc.	185	$5.7x10^9$	+
12	Male	41	Asian	236	$4.4x10^8$	+
13	Male	29	Cauc.	203	$9.9x10^8$	+
14	Female	29	Asian	72	$1.7x10^8$	+

All 14 patients were HBeAg positive at inclusion and were treated with Peg-IFNα for 52 weeks. At the end of therapy 6 patients (case 9 – 14) had serocoverted to HBeAg-negative and this seroconversion was sustained at the end of a follow-up period of 26 weeks. There were 8 non-responders (HBeAg positive, case 1-8) at end of therapy.
Cauc. = Caucasian.

$CD4^+CD25^+$ T Cell isolation. Thawed PBMCs obtained at t=0, t=8, t=52 and t=78 were used for $CD4^+CD25^+$ T cell isolation. $CD4^+$ T cells were isolated from PBMCs by negative selection using the untouched $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+CD25^+$ T cells were isolated from $CD4^+$ T cells using anti CD25-microbeads (Miltenyi biotec). The isolations were performed according to manufacturer's instructions. The $CD4^-$ and the $CD4^+CD25^-$ fraction were pooled and were used as CD25 (Treg)-depleted responder cells. Purity of the cell fractions was determined by flow cytometry analysis with antibodies against CD3, CD4 and CD25. The following antibodies were used: anti-CD3-FITC (clone UCHT1, Immunotech), anti-CD4-PerCP-Cy5.5 (clone SK3, Becton Dickinson) and anti-CD25-PE (clone M-A251, Pharmingen). An isotype matched control antibody was used to determine the level of background staining for the anti-CD25-PE antibody. The $CD4^+CD25^+$ Treg purification method resulted in a Treg fraction containing more than 90% pure $CD4^+CD25^+$ Treg and a Treg-depleted cell fraction containing all other cell types present in PBMCs.

Proliferation assay. Th-cell proliferation was determined as reported previously (22). Isolated PBMCs (and in the Treg-depletion experiments the pooled CD25-depleted cells) of all time points investigated were cultured in triplicate in a concentration of $1X10^5$ cells per well in 100 μl RPMI 1640 (Bio Whittaker, Verviers, Belgium) containing 5% pooled human serum (Dept. of Immunohematology and Bloodbank, Leiden University Medical Center, Leiden, The Netherlands) and penicillin/streptomycin (Gibco, Paisley, UK). The cells were stimulated with 1 μg/ml HBV core antigen (HBcAg) (a kind gift by M van Roosmalen, Biomerieux, Boxtel, The Netherlands), 5 μg/ml Phytohemagglutin (Murex, Paris, France), or not stimulated and cultured for 6 days. After 5 days of incubation the cells were pulsed with 0.25 μCi/well of [^{3}H]thymidine (Radiochemical Centre, Amersham, Little Chalfont, UK). The cells were harvested 16 hours later. Proliferation, was determined by liquid scintillation counting of the harvested cells and expressed as stimulation index (SI). The SI was calculated from the counts per minute found with antigen divided by that found without antigen.

Elispot assay. Detection of IL-10 (Sanquin, Amsterdam, The Netherlands) was performed using ELISPOT kits according to the manufacturer's instructions. Of all time points investigated, cultures of PBMCs and the CD25-depleted cells

were established in triplicate on round bottom 96-wells plates (Corning Inc., New york, USA), in a concentration of $1X10^5$ cells per well in 100 μl RPMI 1640 (Bio Whittaker) containing 5% pooled human serum (Leiden University Medical Center) and penicillin/streptomycin (Gibco). The cells were stimulated with 1 μg/ml HBV core antigen (HBcAg) (M. van Roosmalen, Biomerieux), 5 μg/ml Phytohemagglutin (Murex) (positive control) or medium only (negative control) for 24 hours at 37°C, after which they were transferred into flat bottom anti-IL-10-coated 96-wells plates (Nunc A/S, Roskilde, Denmark and Millipore, Molsheim, France, respectively) for another 24-hours incubation period. Cytokine spots were visualised by biotin-labelled antibodies and their numbers were established by using a Bioreader 3000 from BioSys (Karben, Germany). A response was considered positive if the number of spots was equal to or greater than 2.5 times the background, and the reported HBcAg-specific data is the number of spots counted minus the background level.

Statistics. Treg flow cytometry data, cytokine profiles and proliferation capacity of PBMC obtained from responders and non-responders to antiviral therapy were compared using the Mann Whitney U test. Longitudinal analysis of frequencies of Treg, cytokine profiles and proliferation capacity during the study period, was performed with the Wilcoxon matched pairs signed rank sum test. For these analyses SPSS 11.5 for Windows (SPSS, Chicago, IL) was used. Where applicable data are reported as mean ± standard error of the mean (SEM) or median and range.

Results

Patient characteristics. Table I shows the patient characteristics. At the end of follow-up (t=78) 8 patients had remained HBeAg positive (non-responders), and 6 patients had sustained loss of HBeAg (responders; 43%). At the start of treatment (t=0), there were no significant differences in ALT value or viral load between responders and non-responders. The 8 non-responders had a median ALT of 102 IU/l (range 47-394) versus 168 IU/l (range 72-236) in responders, and the median viral load measured $2.3x10^9$ cp/ml (range $1.6x10^8$-$7.1x10^9$) versus $1.1x10^9$ cp/ml (range $1.7x10^8$-$5.7x10^9$), respectively. At the end of therapy and at the end of follow-up median HBV DNA levels were $1.8x10^8$ cp/ml ($1.4x10^3$-$4.7x10^8$) and $5.3x10^8$ cp/ml ($3.4x10^5$-$4.9x10^9$) in non-responders, vs. $1.0x10^3$ cp/ml (480-$2.9x10^4$) and 473 cp/ml (<373-970) in responders, respectively.

HBV-specific T-cell proliferation is associated with response to Peg-IFNα therapy. A proliferation assay was performed to measure the immune responsiveness of Th-cells to HBcAg longitudinally during the course of the treatment. The pattern of Th-cell proliferation during Peg-IFNα therapy in responders and non-responders differed considerably. At the start of therapy there was no significant difference between responders and non-responders (mean SI = 4.33 ± 0.63 and 3.91 ± 0.38, respectively). However, compared to the start of therapy at t=8 and t=52 weeks in responders the proliferation capacity of Th-cells had increased by 25% and 175% (mean SI = 6.02 ± 0.88 and 16.61 ± 3.70, respectively), whereas in non-responders compared to the start of therapy at t=8 and t=52 weeks the mean proliferation capacity of Th-cells had decreased by 35% and 76%, respectively (mean SI = 2.50 ± 0.19 and 1.00 ± 0.07, respectively, $p < 0.05$) (figure 1).

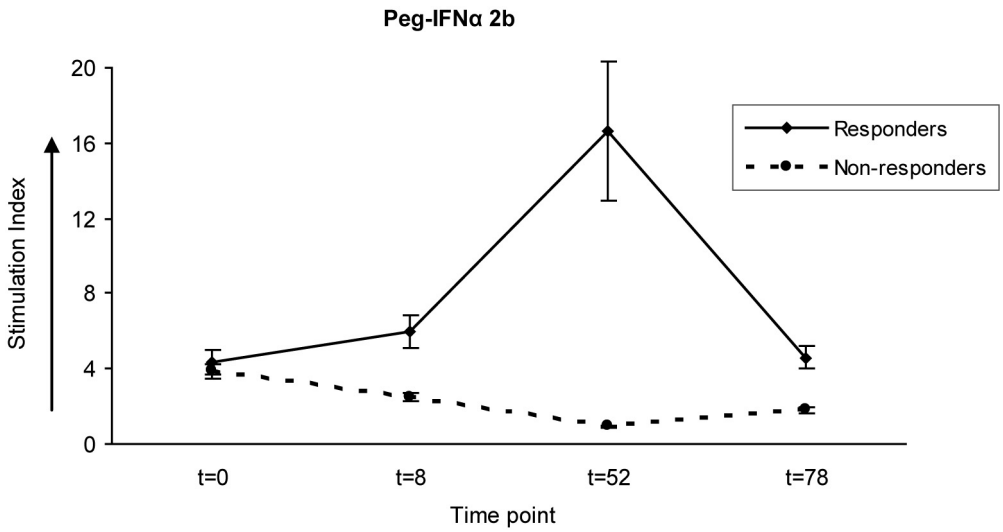


Figure 1. Mean proliferation capacity (± sem) of Th-cells of patients with chronic HBV, obtained at different time points during Peg-IFNα therapy, after stimulation with HBcAg. The time points analyzed were: t=0, t=8, t=52 and t=78 weeks. In non-responders, compared to t=0 at t=8 and t=52 the reduction in stimulation index was significant (p=0.016 at both time points). At 8 and 52 weeks of treatment the differences between responders and non-responders showed a trend towards significance (p = 0.07 and p = 0.10, respectively).

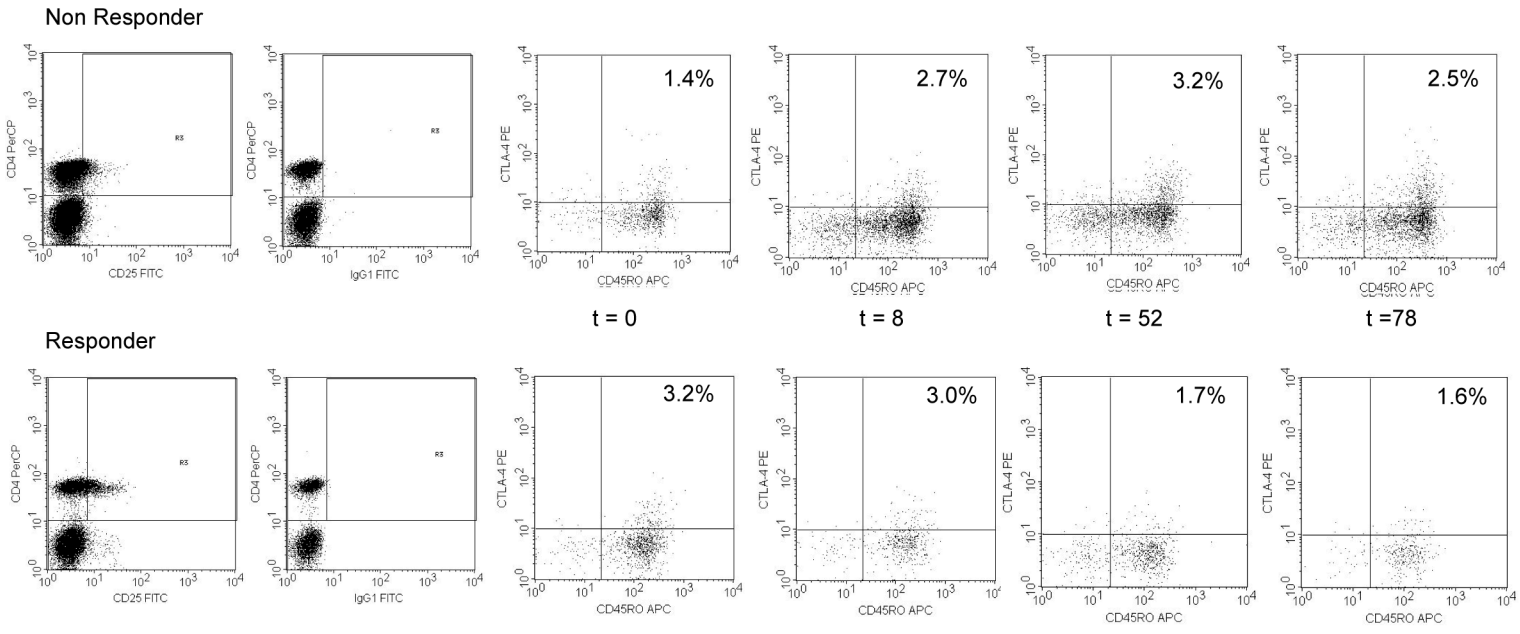
Increased frequencies of CD4+ CD25+ regulatory T cells in non-responders during PEG-IFNα therapy. At all time points investigated we determined the frequencies of CD4+ CD25+ Treg. Based upon several studies

which showed that the $CD4^+CD25^+$ cells with high expression of CD25 are Treg (16,20,23), CD45RO and CTLA-4 were used as additional markers in our flow cytometry experiments to identify this cell population. Representative data as obtained by flow cytometry of PBMCs from a responder and non-responder, are shown in figure 2a. Figure 2b shows the mean frequencies of CD4+ CD25+ Treg in responders and non-responders during treatment. At baseline responders and non-responders had comparable CD4+ CD25+ Treg frequencies (2.21% ± 0.10 vs 2.23% ± 0.19 respectively). At the end of therapy in non-responders the frequency had increased by 74% to a mean of 3.6% ± 0.18 ($p = 0.008$), whereas in responders it had decreased by 20% to a mean of 1.75% ± 0.09 ($p = 0.031$;responders vs. non-responders $p = 0.008$). At the end of follow-up in non-responders the frequency CD4+ CD25+ Treg returned to the baseline level, whereas in responders it further decreased to below baseline (responders vs. non-responders $p = 0.074$).

CD4+ CD25+ Treg express the *forkhead*/winged helix family protein Foxp3, which is a key regulator for the development and function of Treg, and currently the most specific marker for this cell population (15,24,25). The recent availability of an adequate Foxp3 antibody enabled us to retrospectively validate our Treg staining. Of 10 patients (7 of 8 non-responders and of 3 of 6 responders) material was available to determine the Foxp3 expression by CD4+ CD25+ CTLA-4+ cells. Almost all CD4+ CD25+ CTLA-4+ cells expressed the Foxp3 protein (mean percentage Foxp3+ / CD4+ CD25+ CTLA-4+ cells in all patients: 98% ± 0.46; responders vs. non-responders: $p > 0.05$). Figure 3a shows flow cytometry data of a representative patient (case 1).

Retrospectively, in the same 10 patients we also analyzed Foxp3 expression by CD4+ CD25+ cells during the course of treatment (figure 3b). At the start of therapy responders and non-responders had comparable mean frequencies of CD4+ CD25+ Foxp3+ Treg (4.83% ± 0.23 vs. 5.36% ± 1.19 respectively, $p > 0.05$). At the end of therapy in non-responders the frequency of CD4+ CD25+ Foxp3+ Treg had increased significantly by 62% to a mean of 7.83% ± 0.50 ($p = 0.043$). At the end of follow-up this frequency had returned to the baseline level (mean percentage CD4+ CD25+ Foxp3+ Treg 5.94 ± 0.72; t=0 vs. t=78 $p > 0.05$). During and after treatment in responders the frequencies of CD4+ CD25+ Foxp3+ Treg did not change significantly. Figure 3c shows the flow cytometry data of 2 representative patients.

A


Non Responder
CD4 PerCP
CD25 FITC
IgG1 FITC
CTLA-4 PE
CD45RO APC
1.4%
2.7%
3.2%
2.5%
t = 0
t = 8
t = 52
t =78
Responder
3.2%
3.0%
1.7%
1.6%

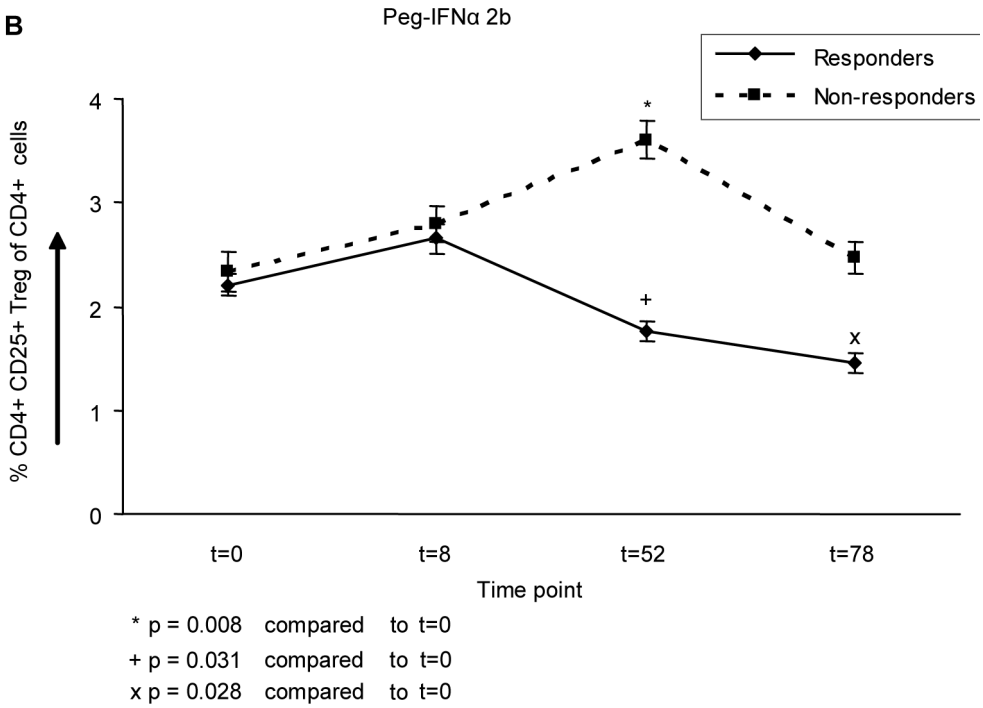
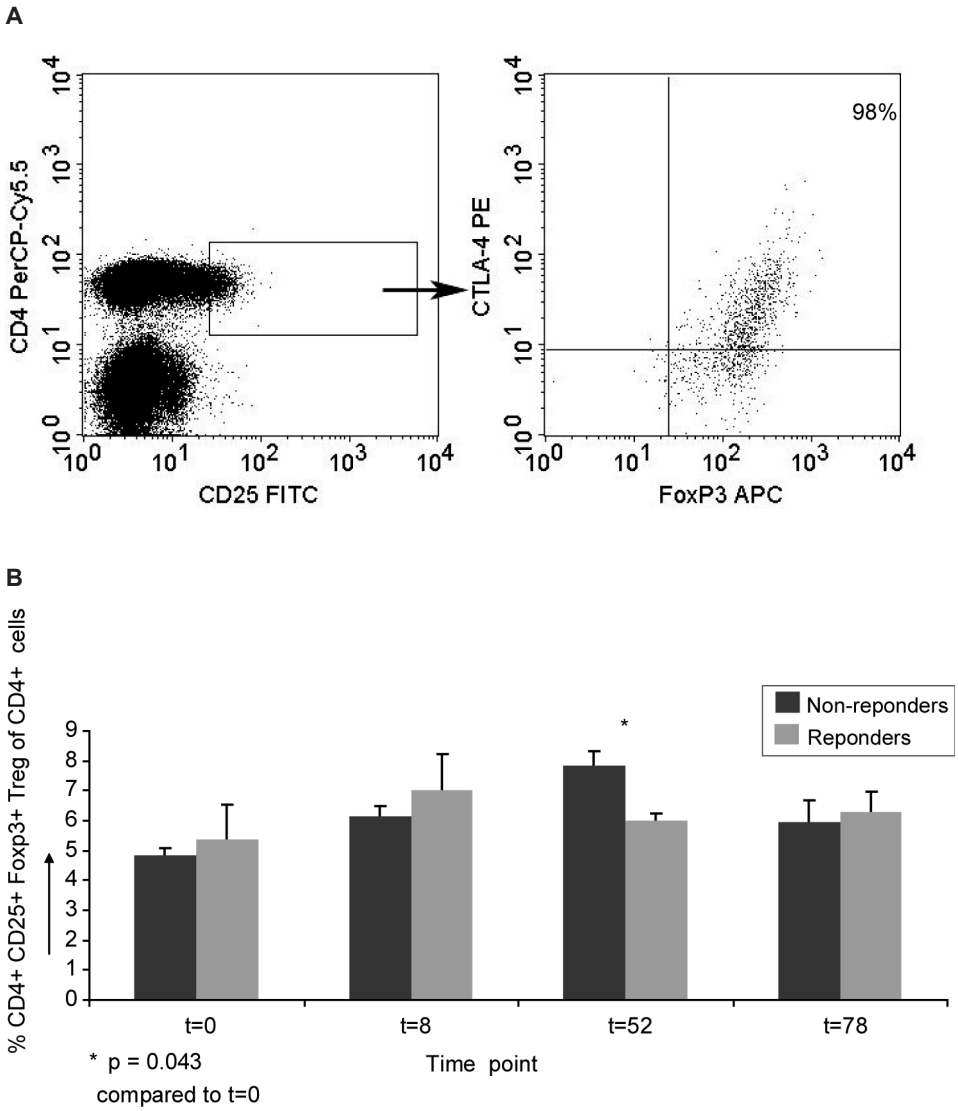


Figure 2. Percentage of Treg (CD4, CD25, CD45RO and CTLA-4 positive cells) within the $CD4^+$ cell fraction, determined by FACS staining on PBMC of 8 non-responders and 6 responders to Peg-IFNα therapy. The time points analyzed were treatment week 0, 8, 52 and 78. **A)** Flow cytometry staining experiment from a representative responder (case 10) and non-responder (case 4) to Peg-IFNα therapy, during the course of the study. Antibodies were used against CD4, CD25, CD45RO and CTLA-4. The gates were positioned using isotype matched control antibodies as shown in the two dot-blots on the left. $CD4^+CD25^+$ cells were analyzed for CTLA-4 and CD45RO expression, and the number in the upper right quadrant of de four dot blots on the right indicates the percentage CTLA-4+ CD45RO+ CD4+ CD25+ cells of total CD4+ cells (Treg). **B)** Mean (± sem) frequencies of Treg (CD4, CD25, CD45RO and CTLA-4 positive cells) during the study. Compared to the baseline level, in non-responders there was a significant increase in the frequency of Treg at the end of therapy, whereas in responders there was a significant decrease. At the end of therapy the frequency of Treg was higher in non-responders than responders ($p = 0.008$). At t=78 in non-responders the frequency had returned to baseline level, whereas in responders it continued to decrease (responders vs. non-responders $p = 0.074$)

CD4+ CD25+ Treg do not account for the increased frequencies of HBV-specific IL-10-producing cells observed in non-responders during Peg-IFNα therapy. We longitudinally studied the frequency of IL-10 producing cells after stimulation with HBcAg *in vitro*. During and after treatment in responders

the frequencies of IL-10 producing cells did not change significantly. However, during treatment in non-responders there was a gradual and significant increase in frequency of IL-10 producing cells from a mean of 22.7 ± 4.0 / $1x10^5$ PBMC at t=0 to a mean of 48 ± 6.7 / $1x10^5$ PBMC at t=52 weeks (p = 0.016). This number returned to the baseline level after cessation of the therapy (figure 4).



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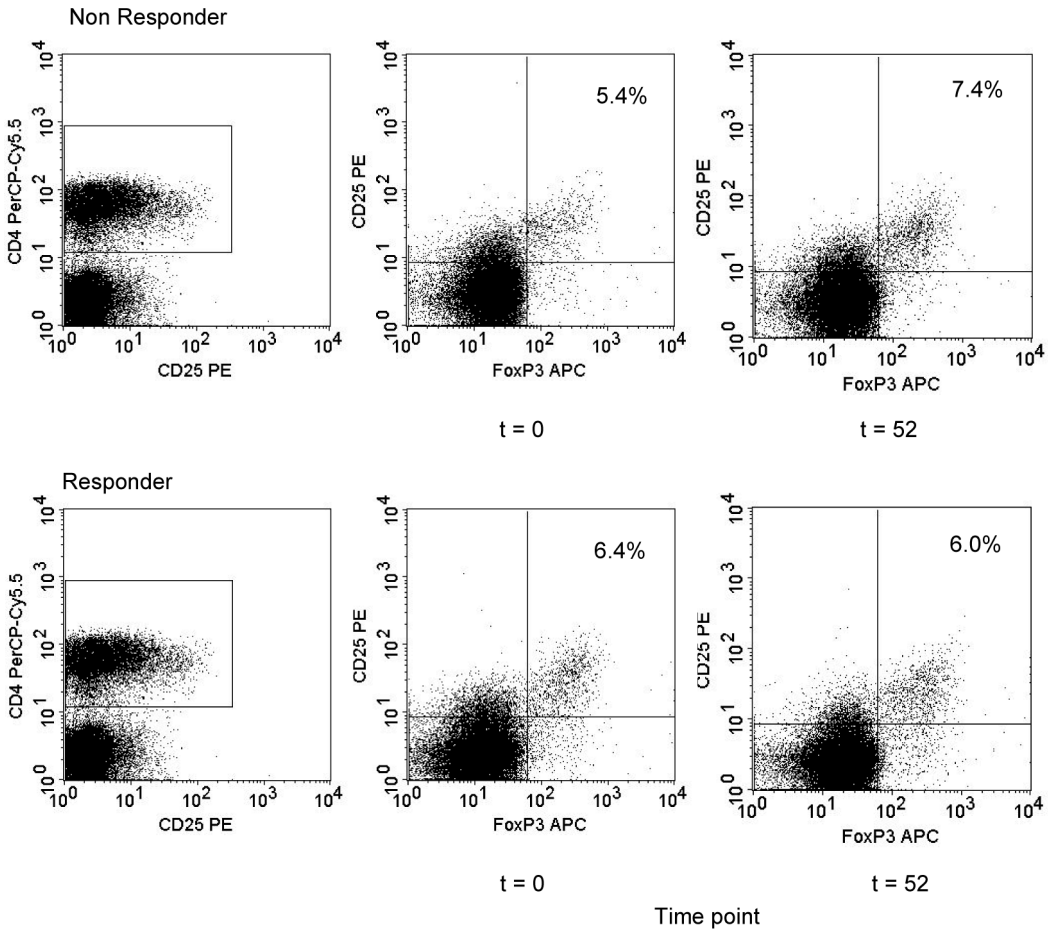

Figure 3. Foxp3 expression by CD4+ CD25+ Treg. **A)** Foxp3 expression by CD4+ CD25+ CTLA-4+ cells at the start of therapy in a representative chronic HBV patient treated with Peg-IFNα (case 1). The number in the right upper quadrant of the right dot-blot indicates the percentage Foxp3+ cells of total CD4+ CD25+ CTLA-4+ cells. Almost all CD4+ CD25+ CTLA-4+ cells expressed the Foxp3 protein. **B)** Mean frequencies of CD4+ CD25+ Foxp3+ cells of total CD4+ cells during therapy. Compared to the start of therapy, in non-responders there was a significant increase in the frequency of CD4+ CD25+ Foxp3+ cells at the end of therapy, which returned to the baseline level in the follow-up period. During and after treatment in responders the frequencies of CD4+ CD25+ Foxp3+ Treg did not change significantly, and there were no significant differences in the frequencies of CD4+ CD25+ Foxp3+ Treg between responders and non-responders. **C)** Flow cytometry staining experiment from a representative responder (case 10) and non-responder (case 7) to Peg-IFNα therapy, at the start and the end of therapy. The number in the upper right quadrant of de two dot blots on the right indicates the percentage CD4+ CD25+ Foxp3+ cells of total CD4+ cells.

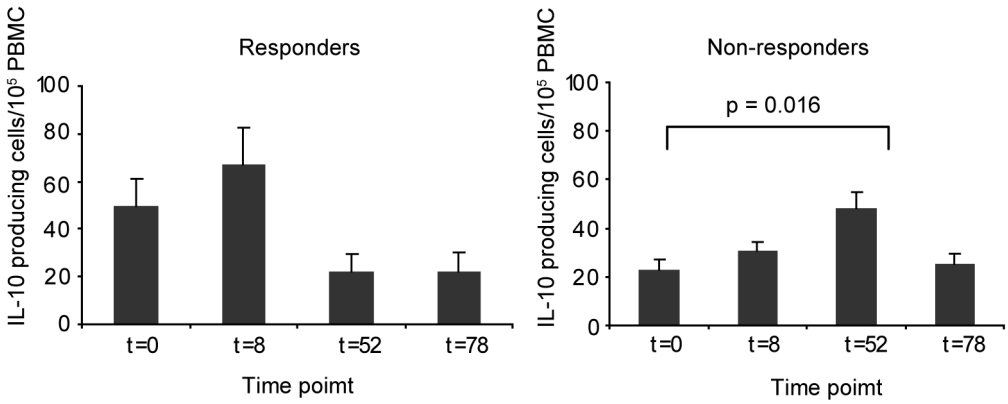

Figure 4. Mean frequencies of IL-10 producing cells (± sem) at treatment week 0, 8, 52 and 78, according to treatment response. In contrast to the responders, during treatment the non-responders showed a significant increase in frequency of IL-10 producing cells. At no time point analyzed there was a significant difference between responders and non-responders.

To analyze whether IL-10 was produced by CD4+ CD25+ Treg, we performed CD4+ CD25+ T cell depletion experiments in four patients (2 responders, 2 non-responders) of which sufficient cells were available. When CD4+ CD25+ T cells were depleted, the HBV-specific proliferation capacity of Th-cells increased (figure 5). This suggests that the CD4+ CD25+ T cell population studied is indeed able to suppress the antiviral immune response, as has been shown before (20,21,26). Interestingly, Treg depletion did not influence the number of IL-10 producing cells.

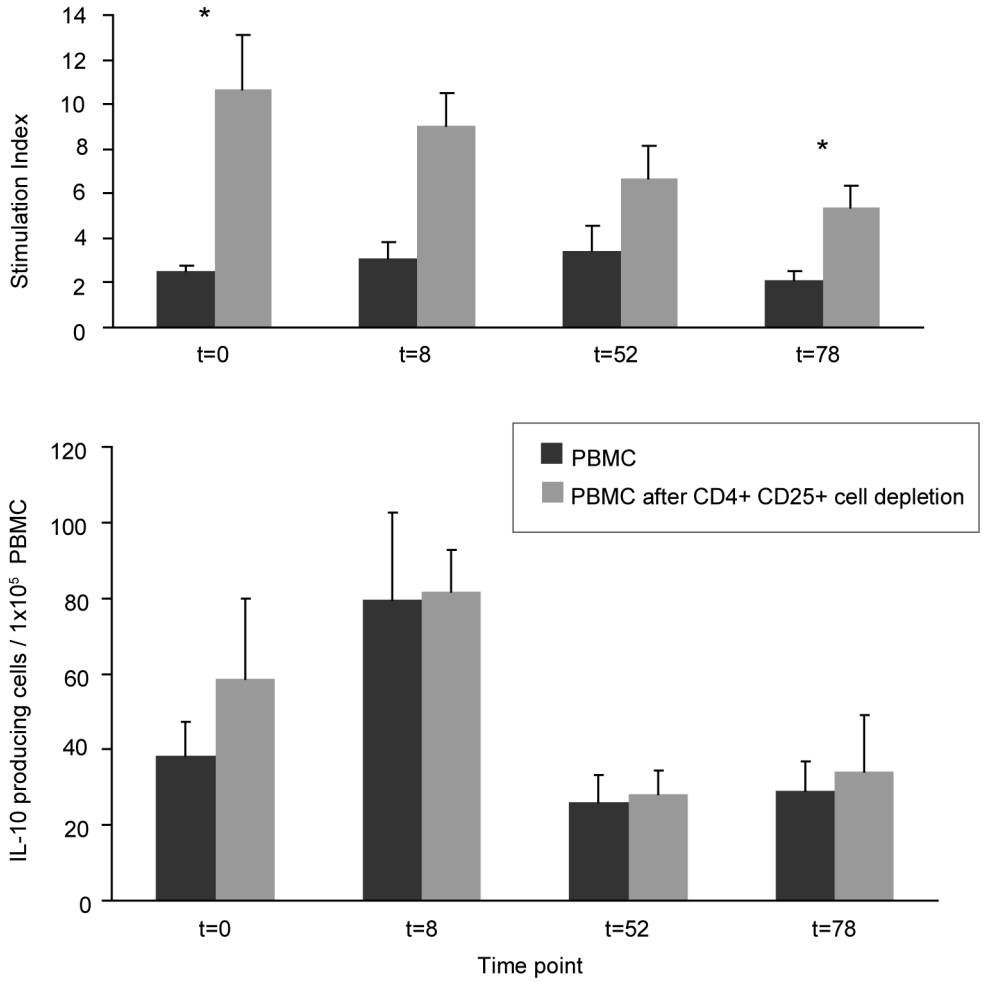

Figure 5. CD4+ CD25+ regulatory T-cell depletion. The effects of CD4+ CD25+ Treg depletion on the proliferation capacity (top) and the frequency of IL-10 (bottom) producing cells in four patients (2 responders, 2 non-responders) after stimulation of PBMC with HBcAg *in vitro.* The bars represent means ± sem of the pooled data. PBMC were depleted of CD4+ CD25+ cells at all time points during the study (week 0, 8, 52 and t=78). Despite the low patient number, there was a trend towards significance with respect to increased proliferation capacity after Treg depletion (* $p \leq 0.1$), whereas the frequencies of IL-10 producing cells was unchanged.

Discussion

Peg-IFNα therapy leads to sustained virological response in a minority of chronic HBV infected patients (9,10). Elucidating the mechanism responsible for treatment failure may result in improved control of the virus infection and better treatment strategies. In this study we have investigated the HBV-specific Th-cell responses in peripheral blood of HBeAg-positive CHB patients treated with Peg-IFNα . Since it was recently shown that patients with chronic HBV infection have a higher percentage of CD4+ CD25+ Treg in their peripheral blood than individuals with a resolved infection, and that these Treg were capable of inhibiting the HBV-specific immune response (20), we have investigated the role of this T-cell population in the outcome of therapy. The results show that during therapy, in the majority of responders there is increased proliferation of Th-cells after stimulation with HBcAg, whereas in non-responders there is a clear decrease. Previously, it has been described that responders to conventional IFNα display a similar increased Th-cell proliferation capacity (27) which may reflect increased Th-cell reactivity to the virus.

At the start of treatment there was no difference in the frequencies of CD4+ CD25+ Treg between responders and non-responders. However, during Peg-IFNα therapy in non-responders there was a significant increase in the frequency of CD4+ CD25+ CD45RO+ CTLA-4+ Treg, whereas in responders this frequency had significantly decreased both at the end of therapy and follow-up. Treg express the Foxp3-gene, which is important in the development and suppressive function of CD4+ CD25+ Treg (24,25). Using a Foxp3 antibody we showed that almost all CD4+ CD25+ CTLA-4+ T-cells express this protein, and thus indeed are a regulatory T-cell population. Furthermore, analysis of Foxp3 expression by CD4+ CD25+ T-cells confirmed that in non-responders but not in responders to Peg-IFNα therapy, during treatment there is an increase in Treg frequency that normalizes to the baseline level after treatment is discontinued. Interestingly, the observed frequencies of CD4+ CD25+ Foxp3+ Treg were higher than the frequencies of CD4+ CD25+ CD45RO+ CTLA-4+ Treg, and not all CD4+ CD25+ Foxp3+ Treg expressed CTLA-4 (data not shown). It was recently shown that anti-CTLA-4 monoclonal antibody treatment blocks CD4+ CD25+ Treg function, suggesting that CTLA-4 has a specific, nonredundant role in the function of these Treg (28). Therefore, in our study the CD4+

CD25+ CD45RO+ CTLA-4+ Tregs may represent the functional subpopulation of CD4+ CD25+ Foxp3+ Treg, explaining the differences in their frequencies, and why at the end of therapy the observed difference in the frequencies of CD4+ CD25+ CD45RO+ CTLA-4+ Treg between responders and non-responders, was absent when comparing the frequencies of CD4+ CD25+ Foxp3+ Treg. It suggests that only in non-responders there is an increase of functional Tregs during Peg-IFNα therapy. We and others (20,21,26) have shown that CD4+ CD25+ Treg are capable of inhibiting the HBV-specific immune response. Therefore, one could hypothesize that as opposed to responders, in non-responders an increment in Treg frequency during Peg-IFNα therapy may negatively influence T-cell reactivity to the virus, as illustrated by the reduced Th-cell proliferation, and contribute to treatment failure.

Additionally, in mice vaccine-induced virus-specific T-cell responses were shown to be suppressed by another major regulatory T-cell population that has been implicated in the control of specific T-cell immunity, namely CD4+ IL-10+ Tregulatory-cells (Tr1-cells) (29, 30). IL-10 could be instrumental in Tr1-mediated suppression of T-cell proliferation and cytokine production in a cell-cell contact independent manner (31). These mouse Tr1-cells, were up-regulated under the influence of IFNαβ (29). Also, human Tr1 cells can be generated in vitro under the influence of exogenous IFNα (32). In our study, during the course of Peg-IFNα therapy we found that in contrast to the responders, the non-responders showed a significant increase in frequency of IL-10 producing cells. IL-10 is important in blocking proinflammatory cytokine production, co-stimulation, MHC class II expression, and chemokine secretion (33), and besides Tr1 cells this cytokine can be produced by monocytes,(34) dendritic cells (DC) (35), and potentially CD4+ CD25+ Treg (36). Increased IL-10 production has previously been described in chronic HBV-patients not responding to ribavirin - IFNα combination therapy (37). This observation suggests a dissociation in IL-10 production in relation to response to therapy. We were unable to show a correlation between the frequencies of CD4+ CD25+ Treg and IL-10 producing cells in the individual patients (data not shown). Furthermore, despite our finding that depletion of the CD4+ CD25+ T-cell population resulted in increased proliferation capacity after stimulation with HBcAg, it did not significantly affect the frequency of IL-10 producing cells measured during the course of the treatment. Also, monocytes were not responsible for the observed IL-10 production since flow cytometry experiments confirmed that these adherent cells were not transferred into the elispot plates after

preincubation with HBcAg (data not shown). Interestingly, recent data suggest that IL-10 produced by virus-specific CD8+ T cells may mediate suppression of the immune response in chronic hepatitis C infection (38). Therefore, in non-responders the immunomodulatory effects of Peg-IFNα therapy may result in an increment in Treg frequency and an up-regulation of IL-10 producing cells, (potentially IFNα-induced Tr1-cells, DC or virus-specific CD8+ T cells), which together may negatively influence T-cell reactivity to the virus, and contribute to treatment failure.

Previous studies conducted in patients with chronic HBV infection suggest that there is a positive correlation between HBV-specific CD4+ CD25+ Treg frequencies in liver and PB, and viremia levels (21,39). In our study, no correlation was found between HBV DNA or ALT levels and the frequencies of Treg and IL-10 producing cells (data not shown). Therefore, the up regulation of these immunoregulatory factors observed in non-responders can most likely not be explained by a change in the levels of HBV DNA .

In conclusion, this study indicates that there may be an important role for regulatory T-cells, in HBV-persistence during and after Peg-IFN therapy. We found that an increase of the frequencies of IL-10 producing cells and CD4+ CD25+ Treg during treatment was inversely correlated to treatment response. In non-responders, these immunomodulatory effects of Peg-IFNα therapy may contribute to non-response to treatment

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Discussion

8

CD4+CD25+ Regulatory T cells (Treg) play a role in the impaired immune response to several different foreign antigens (1-3). They can be distinguished from CD4+ effector T cells by their continuous expression of the activation marker CD25 and the expression of the Treg specific transcription factor FoxP3 (4,5). Treg are capable of inhibiting the effector functions of CD4+, CD8+, NK cells and NKT cells (6-11). Treg can also indirectly inhibit the antibody production by B cells via inhibition of CD4+ T cells (12). Several studies have shown that Treg play an important role in maintaining the peripheral immune tolerance (7,13,14). Furthermore, there is increasing evidence that Treg contribute to the immunological hyporesponsiveness against several pathogens, resulting in chronic infections. The balance between effector T cells and Treg is thought to be of great importance for clearing infections (2,3,15,16). It has been suggested that Treg can be induced through repetitive stimulation of T cells by high concentrations of antigen for extended periods (17). Since Treg are major regulators of the immune response it is very important to investigate their role in a chronic HBV infection.

The role of Treg in the immuno-pathogenesis of chronic HBV

In patients with an acute self limiting HBV infection, a multispecific CD4+ and CD8+ T cell response with a type 1 cytokine profile, is important for control of the infection (18,19). Patients with a chronic HBV infection lack such a vigorous multispecific T cell response (20-23).

In chapter two is shown that chronic HBV patients have an increased proportion of Treg in their peripheral blood compared to individuals who have resolved their HBV infection and compared to healthy controls. These Treg were capable of inhibiting the proliferation and IFN-γ production by CD4+ T cells against HBV core antigen (HBcAg) in a dose dependent manner and depletion of Treg resulted in an increased proliferation and IFN-γ production against HBcAg. Furthermore, HBV e antigen (HBeAg) positive patients had higher percentages of peripheral blood Treg compared to HBeAg negative patients (24). The fact that no correlation is observed between viral load and the proportion of peripheral blood Treg could very well be explained by the large inter-individual differences in the proportion of Treg, which is observed in patients as well as in healthy controls. In another study an increased

proportion of peripheral blood Treg was observed in patients with an active chronic HBV infection, which was defined as a high viral load and an elevated serum alanine aminotransferase (ALT) level (25). In addition, it has been shown that Treg are capable of inhibiting the HBcAg-specific proliferation and IFN-γ production of CD8+ T cells. However in this study no difference in the proportion of Treg between patients and healthy controls was observed (26). It is very difficult to compare this study with the other studies, since FoxP3 was not examined.

Yang *et al* observed the same increase of Treg in patients with a chronic HBV infection compared to healthy controls and people with a resolved HBV infection. They also showed the difference in the proportion of Treg between patients who were still HBeAg positive and patients who have seroconverted to HBeAg and they found a correlation between the proportion of peripheral blood Treg and viral load (27).

Contrary to the data described in chapter two and the other studies mentioned above, in which the proportion of Treg is compared, Yang *et al* had the opportunity to perform flowcytometry with anti-FoxP3 and are the only group to define Treg as CD4+CD25+FoxP3+ (25-27). The fact that we did not observe a correlation between the viral load and the proportion of peripheral blood Treg might therefore be explained by the different patient populations studied as well as the different definition used to define Treg. In our study we defined Treg as cells positive for CD4, CD25, CTLA-4 and CD45RO (24), while Yang et al defined Treg as cells positive for CD4, CD25 and FoxP3 (27). We have compared these two staining methods in four patients over time and observed the same trend in the proportion of positive cells. However, the CD4, CD25, CD45RO, and CTLA-4 antibody combination detected lower percentages of Treg compared to the CD4, CD25 and FoxP3 antibody staining (28). Additional flowcytometry experiments revealed that 97% of the CD4+CD25+CTLA4+ cells expressed FoxP3, but not all FoxP3 positive cells expressed CTLA-4 (28). Since CTLA-4 might have a functional role in the Treg-induced immune suppression, CTLA-4 positive Treg could well represent an activated sub-population of the FoxP3+ Treg (29-31).

In the studies described above, isolated Treg were capable of inhibiting T cell proliferation and IFN-γ production. Additionally, a study with a mice model for DNA vaccination revealed that Treg also had an in vivo effect on HBV-specific T cells. Treg depletion prior to vaccination of the mice resulted in higher numbers of INF-γ

producing cells HBV specific CD8+ T cells with increased avidity (32). A schematic overview of the effect of Treg on the anti-viral immune response during a chronic HBV infection is depicted in figure 1.

Treg are considered to be a potential target for immunomodulatory therapy in patients with a chronic HBV infection. Although immunomodulatory therapy targeting Treg function appears to be a therapeutic approach for chronic HBV, it would probably have some serious side effects. Several studies have shown that Treg play an important role in maintaining the peripheral immune tolerance and therefore a systemic decrease in Treg functioning would probably lead to the development of auto-immune symptoms (7,13,14).

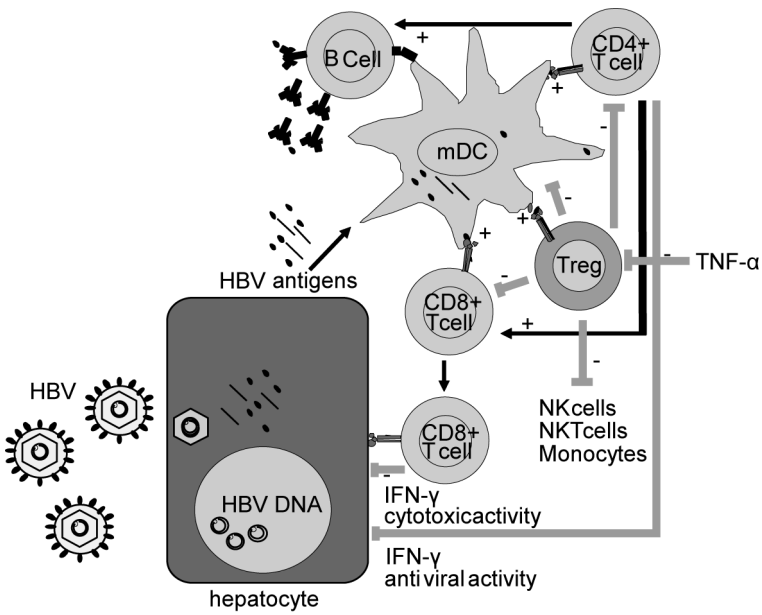

Figure 1. Schematic overview of the different interactions between Treg and the other cells of the immune system, after a hepatocyte is infected with HBV. An arrow accompanied by a plus symbol indicates a stimulatory signal and an arrow accompanied by a minus symbol indicates an inhibitory signal.

Kupffer cells and liver sinusoidal endothelial cells continuously express the immunoregulatory cytokines IL-10 and TGF-β (33). Several studies have shown that TGF-β is important for the induction of Treg (34-37). The local cytokine environment of the liver therefore appears be an ideal environment for Treg induction. Treg induction in the liver draining lymph node has also been described (38). Most studies

concerning HBV and Treg are predominantly focused on peripheral blood Treg, mainly due to the lack of sufficient liver material available for research purposes. In chapter 5 Treg were studied in liver biopsy samples. When patients were divided into two groups, one with high viral load and one with low viral load, the patient group with the high viral load had a higher proportion of intra-hepatic Treg. These data are in line with data of Yang et al using immunohistochemistry (27). The difference between patients with a high viral load and patients with a low viral load was not observed for the proportion of peripheral blood Treg. This corresponds with the results described in chapter 2. The fact that no correlation was observed could be explained by the large inter-individual variation in the proportion of Treg between the different patients.

Treg can be induced through repetitive stimulation of T cells by the presence of high concentrations of antigen for longer periods of time (17). This could be an explanation for the higher proportion of Treg observed in the liver of patients with a high viral load. Another possible cause of the increased proportion of Treg observed in patients with a high viral load is the level of HBV x antigen. HBV x antigen is present in all HBV infected cells. The presence of HBV x antigen is suggested to induce TGF-β production (39). If patients with a high viral load have more HBV x antigen in their hepatocytes, this could result in higher levels of intra-hepatic TGF-β, which could in turn lead to increased Treg induction (34-37).

No correlation was observed in the proportion of intra-hepatic Treg as well as in the proportion of peripheral blood Treg and the amount of liver damage, as defined by ALT levels. Also no relation between liver fibroses and the proportion of intra-hepatic or peripheral blood Treg was observed (Chapter 5 of this thesis). This indicates that the increased proportion of Treg observed in patients with a chronic HBV infection is not a result of liver damage and the presence of self antigen because of this liver damage.

The liver contains a larger population of CD4+CD25-FoxP3+ cells compared to peripheral blood. It has been suggested that Treg can down regulate CD25 depending on their activation status (40). However, a mice study by Nishioka *et al* revealed that CD4+CD25-FoxP3+ cells are also capable of inhibiting the T cell response (41). Since Treg have to be activated to become suppressive (31), this would suggest that the population of CD4+CD25-FoxP3+ cells in the liver is not a population of less activated Treg.

Next to the relatively large population of CD4+CD25-FoxP3+ cells we also detected increased expression of PD-1 on intra-hepatic Treg compared to peripheral blood Treg. The observed higher intra-hepatic PD-1 expression is not specific for Treg, since a higher proportion of the entire population of CD4+ cells in the liver express PD-1. It has recently been shown that PD-1 is involved in the suppressive mechanism of Treg during an allo-immune response and that Treg up regulate PD-1 mRNA after activation (42). The increased expression of PD-1 on intra-hepatic Treg provides additional evidence that these Treg are not less activated compared to peripheral blood Treg. Future studies in which the phenotype of blood and liver Treg is compared in more detail will have to be done. A possible difference in the expression of chemokine receptors and molecules with a possible functional role, such as CTLA-4, could provide further inside in the role of Treg in a chronic HBV infection.

Immunohistochemistry showed that FoxP3 positive cells were predominantly localized in the portal infiltrate and CD8+ T cells were also found outside the portal infiltrates. In a previous study by our group, co-localization of CD8+ and infected hepatocytes was shown (43). Although we did not perform double staining for FoxP3 and HBV core antigen or HBV surface antigen in this study, co-localization of Treg and infected hepatocytes seems very unlikely, since there were almost no FoxP3+ cells observed outside of the portal infiltrates. Since Treg are capable of inhibiting CD8+ T cell function the Treg will probably exert their suppressive effect on CD8+ T cells before the cytotoxic T cells enter the liver parenchyma (26).

Dendritic cells (DC) represent the most potent antigen presenting cell and thus play an important role in the induction of specific T cell responses (44). Myeloid DC (mDC) of chronic HBV patients have been shown to be functionally impaired in their capacity to mature compared to mDC of healthy controls, as shown by a decreased capacity to upregulate co-stimulatory molecules, CD80 and CD86. In addition, patient mDC showed a reduction in their T cell stimulatory capacity, and they exhibited a reduced capacity to produce the immunostimulatory cytokine TNFα (45-47). Up till now, it is unknown which mechanism is responsible for this impaired DC function. Two groups have shown viral particles and RNA replication intermediates within monocyte derived DC of chronic HBV patients (45,48). However Untergasser et al. recently published results showing that although DC were exposed to more than 104 virions per cell, HBV genomic DNA was hardly detected, and no nuclear cccDNA was detected at all (49).

The impaired DC function is thought to be one of the mechanisms responsible for the impaired T cell function observed in patients with a chronic HBV infection. Treg could also be responsible for the impaired DC function. One of the mechanisms by which Treg could interfere with induction of the HBV specific T cell response is by affecting the maturation of DC. Indeed, in chapter 3 is shown that the presence of Treg during the maturation of cell line derived DC results in decreased CD80 and CD86 expression. The data presented in chapter 3 concurs with the data in a paper by Misra *et al*. They also demonstrated that DC are impaired in their T cell stimulatory capacity after culture with Treg (50). The stronger increase observed when the DC are co cultured with responder T cells could be due to IFN-γ produced by the responder T cells. IFN-γ increases the expression of maturation markers on DC and increases their stimulatory capacity (51). The interaction between Treg and DC might be one of the reasons why functionally impaired DC are observed in patients with a chronic HBV infection.

There has been a lot of discussion whether Treg mediated suppression is antigen specific. Thornton et al have shown that Treg have to be activated through their T cell receptor to become suppressive and that after activation Treg exert their suppressive effect in a non antigen specific dose dependent manner (31,52). In most studies a polyclonal stimulus for T cell activation (anti-CD3) or an allogenic stimulus with foreign MHC is used. In vivo such a stimulus will not occur and T cells will only be activated upon recognition of a specific antigen presented by an antigen presenting cell. In chapter 4 we describe a relatively stronger suppressive effect on the response to HBcAg as compared to the response to tetanus toxin (53). Additionally, we have demonstrated in chapter 2 that depletion of Treg did not enhance the anti tetanus toxin response (24). These results could indicate that the proportion of Treg, which are activated upon recognition of HBcAg, is higher than the proportion of Treg which are activated upon recognition of tetanus toxin. Furthermore, the existence of HBV surface antigen specific Treg has been shown in a recent paper (54).

The precise mechanism of suppression by Treg is not known, although it appears that in vitro, suppression by Treg is cell contact dependent (2,55). Treg can secrete IL-10 and TGF-β after activation (2,7,56), however it was not possible to abrogate Treg mediated suppression of the HBV-specific response by means of IL-10 or TGF-β neutralization. This concurs with data from earlier in vitro studies in

which neutralization of these two cytokines had no effect on the suppression of T cells stimulated with anti- CD3 and anti-CD28 (52,55).

Tumor necrosis factor (TNF)-α plays an important role in the control of viral infections. It is involved in the recruitment and activation of macrophages, it can polarize the T cell response towards the development of antiviral effector functions and it has a direct antiviral effect (57,58). TNF-α is essential for the proliferation of HBV-specific cytotoxic T cells (59). Neutralization of the TNF receptor during therapy with Infliximab for Crohn's disease, resulted in patients in (re) activation of hepatitis B virus (60,61). Several interactions between TNF-α levels and Treg functioning and Treg numbers have been described. It has recently been shown that Infliximab enhances the suppressive capacity of Treg and that anti-TNF-α therapy induces a distinct Treg population in patients with rheumatoid arthritis via a TGF-β dependent mechanism (37,62,63). Pre-incubation of Treg with TNF-α decreased the suppressive capacity of the Treg in assays with polyclonally activated T cells. (62). In chapter 4 is shown that TNF-α can also partially abrogate Treg mediated suppression of antigen specific proliferation. Additional TNF-α as well as pre-incubation of Treg with TNF-α resulted in a partial abrogation of the suppressive effect of Treg isolated from chronic HBV patients on the proliferation to HBcAg. The fact that Kasahara et al did not observe cytotoxic T cell proliferation in the absence of TNF-α could be caused by Treg mediated suppression, since is has been shown that Treg are capable of inhibiting the proliferation of HBV-specific CD8+ T cells (26,59).

FoxP3 expression levels by Treg are thought to correlate to their suppressive capacity (64). While Valencia et al did observe a decrease in FoxP3 expression after incubation of Treg with TNF, we could not reproduce their results (53,62). The mechanism by which TNF-α affected the suppressive capacity of Treg was not by inducing cell death or apoptosis in the isolated Treg fraction (53).

TNF-α appears to be a key mediator in the chronicity of HBV, it can partially abrogate Treg mediated suppression of HBV specific T cells, is essential for the proliferation of HBV-specific cytotoxic T lymphocytes (59), has a direct anti-viral effect (57) and can induce DC maturation (65). Since, TNF-α is such an important cytokine during a chronic HBV infection, TNF-α might be a target for future immunotherapeutic strategies.

The immune response and Treg during therapy

Previous studies have shown that a decrease in the viral load caused by antiviral treatment results in a transiently increased HBV-specific immune response by CD4+ and CD8+ T cells (66-68). The mechanism responsible for this partial restoration of the immune response remained unclear in these studies. A partial explanation for this restoration is the recovery of DC function caused by the reduced viral load and serum concentration of HBsAg (46,69).

It has been suggested that Treg can be induced through a repetitive stimulation of T cells by the presence of high concentrations of antigen for longer periods of time, which could theoretically occur in chronic HBV patients with a high viral load and that Treg have a high turnover (17,70). In chapter 6 we observed an increase in HBcAg specific proliferation and IFN-γ production during adefovir induced viral load reduction. This increased responsiveness coincided with a decrease in the proportion of peripheral blood Treg and was not caused by a direct effect of the active metabolite of adefovir (28). The increased DC functioning observed during viral load reduction as well as the increased T cell response during viral load reduction, could be a result of a decrease in the proportion of peripheral blood Treg (Figure 2) (46, 66-68). Chapter 6 shows that the response to the control antigen (tetanus toxin) was also increased. This could be explained by a decrease in activated HBV-specific Treg. Treg have to be activated through their T cell receptor, but once activated the suppression by Treg is non-specific (13,31). Therefore, the decrease in viral load will result in less HBV-specific activated Treg able to inhibit the response of non HBV-specific T cells as well (28).

During adefovir treatment the number of IL-10 producing cells did not decrease. The lack in decrease of cells producing this immunoregulatory cytokine could be one of the reasons that antiviral therapy does not lead to a sustained antiviral response. It also suggests that Treg are not solely responsible for the hyporesponsiveness in chronic HBV patients. Another regulatory T cell type, besides CD4+CD25+ Treg, has been described. These so called T regulatory type 1 cells (Tr1) are CD4 positive cells and their suppression is mediated through an IL-10 dependent mechanism (71). These Tr1 cells also play a dominant role in the latent immune response against Epstein Bar virus (72) and could be responsible for

part of the IL-10 production by PBMC cells observed in patients with a chronic HBV infection (73). Since the viral load reduction caused by adefovir is not sufficient to restore the antiviral immune response, as depicted by the unchanged production of the immunoregulatory cytokine IL-10, combination therapy with antiviral drugs and immunomodulatory therapy may be more successful in perpetuating the antiviral immune response after treatment discontinuation.

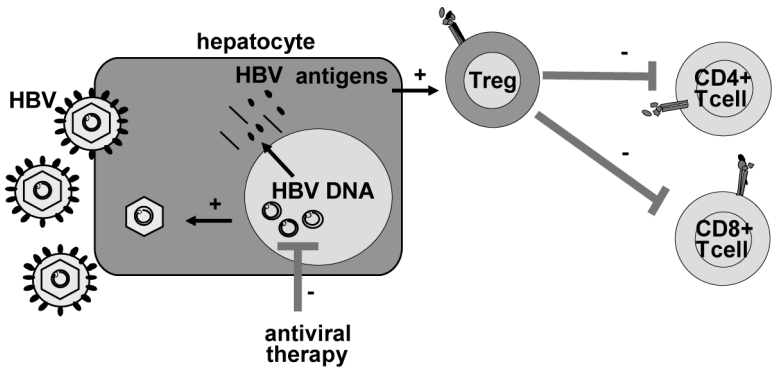

Figure 2. Schematic overview of the consequences of antiviral therapy on Treg and the antiviral response. An arrow accompanied by a plus symbol indicates a stimulatory signal and an arrow accompanied by a minus symbol indicates an inhibitory signal. Antiviral therapy results in a decreased viral load. This decrease in viral load will result in less Treg induction and therefore less inhibition of CD4+ and CD8+ T cell function.

Immunomodulatory therapy for HBV with pegylated IFN-α results in seroconversion for HBeAg in only 36% of the patients (74,75). In Chapter 7 the immune response during therapy with pegylated IFN-α was studied. Compared to non-responders responders displayed an increased HBV-specific T cell proliferation. While there was no difference in the frequencies of Treg between responders and non-responders at the start of treatment, a significant increase in the frequency of peripheral blood Treg was observed in non-responders during treatment. In contrast to the responders, the non-responders showed a significant increase in frequency of IL-10 producing cells. Treg depletion resulted in increased proliferation capacity and increased frequencies of HBV-specific INFγ-producing cells, but did not affect the frequency of IL-10 producing cells measured during the course of the treatment (76). The increase in the proportion of Treg during therapy in non-responders might play

a role in the failure of IFN-α treatment in those patients. The mechanism responsible for the induction of Treg during IFN-α therapy remains to be investigated. Since, it has been demonstrated that TGF-β is involved in the induction of Treg in vitro, this cytokine could play an important role in this mechanism. It would be interesting to compare TGF-β production in responders and non-responders to IFN-α therapy in future studies.

Concluding remarks

The T cell response in patients with chronic HBV infection has been extensively studied. However, because of the complexity of the immune system, it is very likely that a combination of several different shortcomings of the immune systems is responsible for chronicity of an HBV infection. The last few years it has become clear that immunoregulatory cells are involved in the mechanism responsible for the impaired immune response during a chronic HBV infection. The results of the studies described in this thesis show that Treg are important mediators of the impaired immune response in patients with a chronic HBV infection, possibly by affecting the antigen presenting function of DC. IL-10 and TGF-β were not involved in the mechanism of suppression by Treg, however both appear to play a role in the impaired immune response. TGF-β because of it has a potential role in Treg induction and IL-10 as a regulatory cytokine secreted by Tr1 cells or DC. Since TNF-α can affect Treg function and has a direct anti-viral effect, the balance between the proportion of Treg and the TNF-α production might be of critical importance for the outcome of an HBV infection (Figure 3).

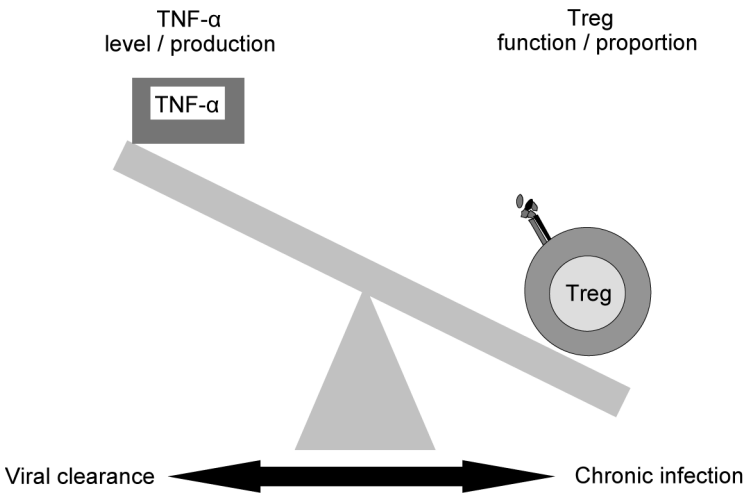

Figure 3. The balance between the proportion of Treg and/or Treg functioning and the TNF-α production could be an important factor in determining whether the patients immune system is capable of clearing the virus.

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Summary

Worldwide 400 million people suffer from chronic hepatitis B virus (HBV) infection and approximately 1 million people die annually from HBV-related disease. To clear HBV, an effective immune response, in which several cell types and cytokines play a role, is important. It is known that patients who develop a chronic infection lack the vigorous multi-specific T cell response with a type 1 cytokine profile necessary to clear the virus. One cell type that could play a role in the absence of an adequate T cell response are CD4+CD25+ regulatory T cells (Treg). These Treg are capable of inhibiting the adaptive T cell response.

In chapter 2 of this thesis the proportion of Treg in the peripheral blood of patients with a chronic HBV infection, individuals with a resolved HBV infection and healthy controls was compared. Patients with a chronic HBV infection showed an increased proportion of peripheral blood Treg compared to the two other groups. Depletion of Treg resulted in an enhanced *in vitro* response to HBV core antigen (HBcAg). Reconstitution of Treg-depleted cells with isolated Treg showed that Treg inhibited the proliferation and interferon (IFN)-γ production to HBcAg in a dose dependent manner. Therefore, Treg appear to be a mediator of the impaired anti-viral response observed in patients with a chronic HBV infection.

One of the proposed mechanisms by which Treg could inhibit the HBV specific T cell response is by affecting antigen presenting cells via down regulation of co-stimulatory molecules. Chronic HBV patients are known to have functionally impaired dendritic cells (DC), an important population of antigen presenting cells. In chapter 3 it is shown that Treg are capable of inhibiting the maturation of DC. Co-culture of DC, derived from a cell line, with Treg during DC maturation resulted in DC with lower expression of the co-stimulatory molecules CD80 and CD86.

Tumor necrosis factor (TNF)-α plays an important role in the control of viral infections. Treg can express the TNF receptor type II. However, no difference was observed in the expression of TNF receptor type II on Treg of chronic HBV patients versus healthy controls. Chapter 4 shows that TNF-α was capable of directly affecting the suppressive capacity of Treg. FoxP3 is thought to correlate with the suppressive capacity of Treg, however the inhibition of the suppressive capacity of Treg by TNF-α was not caused by a down regulation of the FoxP3 expression of the Treg.

Thus far, studies have predominantly focussed on peripheral Treg, however for HBV the liver is the primary site of infection. Therefore chapter 5 is dedicated

to intra-hepatic Treg. Patients with a high viral load showed a higher proportion of intra-hepatic Treg, as compared to patients with a low viral load. This difference was not observed for peripheral blood Treg. There was no relation between previous liver damage or liver inflammation and the presence of Treg. The increased proportion of intra-hepatic Treg observed in patients with a high viral load, suggests that Treg play an important role in HBV tolerance and persistence of viral replication.

It has been suggested that Treg can be induced by repetitive stimulation with high concentration of antigen and that Treg have a high turnover *in vivo.* Consequently, the effect of treatment on the percentage of Treg and the antiviral T cell response has been studied in chapter 6 and 7. Chapter 6 showed that therapy induced viral load reduction by adefovir, an inhibitor of viral replication, resulted in a decrease in the percentage of peripheral blood Treg. This decrease in Treg coincided with an increase in the HBcAg specific IFN-γ production and proliferation. This increased response was not caused by a decrease in the production of the immuno-regulatory cytokine IL-10. The observed effect was not caused by a direct effect of adefovir on the PBMC. Although a decrease in Treg was observed, none of the patients cleared their HBV infection. This indicates that Treg are not solely responsible for the impaired anti-viral immune response in patients with a chronic HBV infection.

Treatment with IFN-α is effective in only one-third of chronic HBV patients. Thus far, the reason for this low response rate in unknown. In chapter 7, the role of Treg in the treatment failure in non-responders to IFN-α therapy, was investigated. At the start of treatment there was no difference in the frequencies of Treg between responders and non-responders. During therapy, the frequency of peripheral blood Treg increased in non-responders, while responders showed a decrease. In addition non-responders showed a significant increase in frequency of IL-10 producing cells. The results of our studies show that Treg are important mediators of the impaired immune response in patients with a chronic HBV infection, possibly by affecting the antigen presenting function of DC. The proportion of Treg is affected by the viral load. Since TNF-α can affect Treg function and has a direct anti-viral effect, the balance between the proportion of Treg and the TNF-α production might be of critical importance for the outcome of an HBV infection.

Samenvatting

Wereldwijd lijden er circa 400 miljoen mensen aan een chronische infectie met het hepatitis B virus (HBV) en jaarlijks sterven er ongeveer 1 miljoen van hen aan de gevolgen van deze chronische infectie. Om HBV te klaren, is een sterke multispecifieke T cel reactie nodig met een type 1 cytokine profiel. Bij patiënten die een chronische HBV infectie ontwikkelen is deze reactie te zwak of zelfs helemaal afwezig. CD4+CD25+ Regulatoire T cellen (Treg) zouden een rol kunnen spelen in deze verminderde immuun reactiviteit, omdat ze in staat de T cel respons te onderdrukken.

In hoofdstuk 2 van dit proefschrift is beschreven dat patiënten met een chronische HBV infectie een hoger percentage Treg in hun bloed hebben in vergelijking met gezonde controles en mensen die HBV geklaard hebben. Depletie van Treg resulteerde in een verbeterde *in vitro* response tegen HBV core antigen (HBcAg). Verder laat dit hoofdstuk zien dat Treg de HBV specifieke respons op een dosis afhankelijke manier kunnen remmen. Treg lijken dus een belangrijke rol te spelen in de verminderde antivirale immuun respons bij patiënten met een chronische HBV infectie.

Tot dusver is het onduidelijk hoe Treg precies de immuun respons kunnen onderdrukken. Eén van de mogelijkheden is dat ze de antigeen presenterende functie van dendritische cellen (DC) remmen. Er is reeds bekend dat DC van patiënten met een chronische HBV infectie minder goed functioneren. In hoofdstuk 3 is beschreven dat Treg in staat zijn om de maturatie van DC te remmen. De aanwezigheid van Treg tijdens DC maturatie resulteerde in een lage expressie van de co-stimulatoire moleculen CD80 en CD86.

Tumor necrosis factor (TNF)-α is een belangrijke cytokine voor antivirale immuniteit. TNF receptor type II komt tot expressie op Treg. Hoofdstuk 4 laat zien dat TNF-α de suppressieve capaciteit van Treg kan verminderen. TNF-α had een direct effect op de Treg. In de literatuur is beschreven dat de FoxP3 expressie van Treg is gecorreleerd aan de suppressieve capaciteit, echter incubatie met TNF-α had geen effect op de FoxP3 expressie van Treg. Verder laat dit hoofdstuk zien dat er geen verschil is de expressie van TNF receptor type II op Treg van chronisch HBV patiënten en gezonde controles.

Tot nu toe zijn de meeste studies naar de rol van Treg in de immunopathogenese van HBV gericht op Treg geïsoleerd uit perifeer bloed. HBV infecteert echter de lever, daarom zijn in hoofdstuk 5 intra-hepatische Treg bestudeerd. Het percentage intra-

hepatische Treg was verhoogd bij patiënten met een hoge virale load, vergeleken met patiënten met een lage virale load. Dit verschil werd niet gevonden voor het percentage Treg in het perifere bloed. Er werd geen correlatie gevonden tussen het percentage Treg en de hoeveelheid leverontsteking of fibrose. De correlatie tussen virale load en het percentage Treg suggereert dat Treg een belangrijke rol spelen in de tolerantie tegen het virus en de persisterende virus replicatie.

In de literatuur is beschreven dat Treg geïnduceerd kunnen worden door het herhaaldelijk stimuleren met grote hoeveelheden antigen en dat Treg een korte levensduur hebben. In hoofdstuk 6 is beschreven dat reductie in virale load, middels antivirale therapie met adefovir, resulteerde in een afname van het percentage Treg in het perifere bloed. Tegelijkertijd met deze afname in Treg werd een toegenomen HBcAg specifieke proliferatie en interferon (IFN)-γ productie gevonden. De toegenomen HBV-specifieke respons werd niet veroorzaakt door een afname in de productie van het immuno-regulatoire cytokine IL-10. Ondanks dat er een daling in het percentage Treg werd gezien tijdens adefovir therapie, kon geen van de patiënten het virus klaren. Treg zijn dus niet de enige cellen die verantwoordelijk zijn voor de verminderde antivirale respons die een chronische HBV infectie in stand houdt.

Behandeling met IFN-α is slechts in één derde van de chronische HBV patiënten effectief. De reden hiervoor is onduidelijk. In hoofdstuk 7 is onderzocht of Treg een rol spelen bij het falen van IFN-α therapie. Bij aanvang van de behandeling was het percentage perifere bloed Treg vergelijkbaar tussen patiënten die uiteindelijk wel en patiënten die niet reageerden op IFN-α therapie. Tijdens de therapie nam het percentage Treg in patiënten die niet reageerden op therapie toe. Dit gebeurde niet bij patiënten die wel reageerden. Bij de patiënten die niet reageerden op therapie werd bovendien een toename van het aantal IL-10 producerende cellen gevonden. Deze bevindingen suggereren dat zowel CD4+CD25+ Treg als IL-10 producerende cellen een rol spelen in het al dan niet reageren op IFN-α therapie.

De resultaten van onze studies laten zien dat Treg een belangrijke rol spelen in de verminderde antivirale response in patiënten met een chronische HBV infectie, mogelijk via de inductie van onrijpe, niet goed functionerende DC. Het percentage Treg wordt beïnvloed door de virale load. Omdat TNF-α een direct effect op zowel Treg als de virale load heeft, zou de balans tussen de hoeveelheid Treg en de hoeveelheid TNF-α die geproduceerd word een grote invloed kunnen hebben op de uitkomst van een HBV infectie.

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Jeroen Stoop

Curriculum Vitae

De auteur van dit proefschrift werd op 22 augustus 1979 geboren in Rotterdam. In 1998 behaalde hij het VWO diploma op het Emmaus College te Rotterdam. In aansluiting hierop studeerde hij Medische Biologie aan de Universiteit Utrecht. Tijdens deze studie liep hij stage bij de afdeling Virologie van de Faculteit Diergeneeskunde, waar hij onderzoek deed naar Muis Hepatitis Virus onder begeleiding van Prof.dr. P.J.M. Rottier en Dr. C.A.M. de Haan en de afdeling Immunologie van het Universitair Medisch Centrum Utrecht waar hij werkte met een phage antibody display library onder begeleiding van Prof.dr. T. Logtenberg en Dr. R.M. Zwijsen. In april 2003 behaalde hij hier zijn doctoraal examen. Direct aansluitend trad hij in dienst als promovendus op de afdeling Maag-, Darm- en Leverziekten van het Erasmus MC te Rotterdam om daar onder begeleiding van Prof.dr. H.L.A. Janssen en Dr. R.G. van der Molen te werken aan het in dit proefschrift beschreven onderzoek. Sinds 1 juli 2007 werkt hij aan Immuun regulatie in een muis model voor reumatoïde artritis in de Musculoskeletal Research group van de Universiteit van Newcastle onder begeleiding van Dr. C.U.M. Hilkens en Prof.dr. J.H. Robinson.

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