Research Article

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Restoration of T cell function in chronic hepatitis B patients upon treatment with interferon based combination therapy

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Background & Aims: Chronic hepatitis B virus (HBV) infection is characterized by functional impairment of HBV-specific T cells. Understanding the mechanisms behind T cell dysfunction and restoration is important for the development of optimal treatment strategies.

Methods: In this study we have first analysed the phenotype and function of HBV-specific T cells in patients with low viral load (HBV DNA <20,000 IU/ml) and spontaneous control over the virus. Subsequently, we assessed HBV-specific T cells in patients with high viral load (HBV DNA >17,182 IU/ml) treated with peginterferon/adefovir combination therapy who had various treatment outcomes.

Results: HBV-specific T cells could be detected directly *ex vivo* in 7/22 patients with low viral load. These showed an early differentiated memory phenotype with reduced ability to produce IL-2 and cytotoxic molecules such as granzyme B and perforin, but with strong proliferative potential.

In a cohort of 28 chronic hepatitis B patients with high viral load treated with peginterferon and adefovir, HBV-specific T cells could not be detected directly *ex vivo*. However, HBV-specific T cells could be selectively expanded *in vitro* in patients with therapy-induced HBsAg clearance (HBsAg loss n = 7), but not in patients without HBsAg clearance (n = 21). Further analysis of HBV-specific T cell function with peptide pools showed broad and efficient antiviral responses after therapy.

Conclusions: Our results show that peginterferon based combination therapy can induce HBV-specific T cell restoration. These findings may help to develop novel therapeutic strategies to reconstitute antiviral functions and enhance viral clearance.

Keywords: Hepatitis B; Combination therapy; HBV-specific T cells; Adaptive immunity; HBsAg loss.

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; CHB, chronic hepatitis B; HBeAg, hepatitis B e antigen; PegIFN, peginterferon alpha 2a; NUC, nucleot(s)ide analogues; HLA, human leucocyte antigen; ALT, alanine aminotransferase; LVL, low viral load; HVL, high viral load; CR, combined response; NR, non-response; CMV, cytomegalo virus; PBA, phosphate buffer/albumin; PBS, phosphate buffered saline; FACS, Fluorescence-Activated Cell Sorting; PMA, phorbol 12-myristate 13-acetate; LFU, long-term follow-up.

Introduction

Patients who are chronically infected with hepatitis B (CHB) are at an increased risk of liver related morbidity and mortality. Treatment of these patients is aimed at prevention of progression to advanced liver disease [1–3]. Ideally, hepatitis B surface antigen (HBsAg) loss and development of anti-HBs antibodies is achieved upon treatment. This outcome is considered the closest to cure and is associated with complete and durable control of the infection.

Available data on acute, self-limiting HBV infection show strong and multispecific HBV-specific T cell responses [4,5]. In patients with low viral load (LVL) CHB, hepatitis B virus (HBV) core-specific T cells can be detected directly *ex vivo* [5–7]. However, not much is known about the exact phenotype and function of these cells. In patients with high viral load (HVL) and active disease, low HBV-specific immune responses are found. HBV is thought to have evolved several mechanisms to avoid the development of an effective immune response [4,8–10]. Repetitive T cell receptor stimulation by persistently high hepatitis B antigen (HBsAg and HBeAg) levels is believed to play a role in T cell exhaustion [11].

Treatment options for patients with chronic active hepatitis B consist of peginterferon alpha 2a (PegIFN) and nucleot(s)ide analogues (NUC). The *in vitro* proliferative capacity of HBV-specific T cells can be restored upon successful treatment with NUCs, which inhibit viral replication [12–14]. Recent data from CHB patients treated with PegIFN did not show restoration of HBV-specific T cells upon successful treatment, as these cells remained at low frequencies during and after treatment [15,16].

In this study we first performed an extensive analysis of the phenotype and function of HBV-specific CD8+ T cells in patients with CHB with a LVL. These patients represent a unique CHB

Research Article

subpopulation because they spontaneously control the virus without the need for treatment and in general have minimal or no liver damage. Next, we longitudinally investigated HBV-specific CD8+ T cells in patients with active chronic HBV infection and a HVL, who were treated with combination therapy of PegIFN and adefovir. HBV-specific CD8+ T cell responses were compared in patients with HBsAg loss upon therapy *vs.* patients who did not respond to therapy. Our results reveal that combination therapy can induce reconstitution of HBV-specific T cells in patients with HBsAg loss. These data show that HBV-specific T cell activation and differentiation can be restored in CHB patients treated with PegIFN and adefovir and foster the development of combination therapy strategies to enhance viral clearance.

Patients and methods

In order to achieve optimal reactivity with HBV-tetramers and genotype-specific peptides, a selection of CHB patients was made based on viral genotype (genotype A, D or E) and HLA-type (HLA-A2 positivity).

Twenty-two out of the 80 non-treated HBeAg negative patients with a LVL (alanine aminotransferase (ALT) <2× upper limit of normal (ULN) and HBV DNA <20,000 IU/ml) that visited our outpatient clinic, were selected for analysis in the present study (Table 1; Supplementary Fig. 1).

Ninety-two patients with HVL and active CHB (44 HBeAg positive and 48 HBeAg negative, HBsAg positivity >6 months, normal or increased (ALT) <10 × ULN and HBV DNA >17,182 IU/ml (>100,000 copies/ml)) were part of a clinical trial in which they received PegIFN alpha 2a (Pegasys®; Hoffman La Roche, Basel, Switzerland) 180 mg subcutaneously once a week and adefovir dipivoxil (Hepsera®; Gilead Sciences, Foster City, CA, USA) 10 mg daily for 48 weeks and were subsequently followed-up for a period of 144–196 weeks [17], (Table 1; Supplementary Fig. 1).

Response definitions of the clinical study, defined at week 72 (6 months after treatment) and follow-up (week 144–196) were as follows (with patient numbers included in the present study):

HBsAg loss (n = 7): persistently undetectable HBsAg combined with undetectable HBV DNA or HBV DNA <20 IU/ml. For HBeAg positive patients this also included HBeAg seroconversion (HBeAg loss with development of anti-HBe antibodies). Two patients had very low HBsAg levels at week 72 and lost HBsAg at week 84 and 144, and were therefore included in this analysis. All patients developed anti-HBs antibodies.

Combined response (CR) (n = 7): HBV DNA <17,182 IU/ml combined with persistently normal ALT values (<ULN) but with still detectable HBsAg. For HBeAg positive patients this also included HBeAg seroconversion.

Non-response (NR) (n = 14): HBV DNA >17,182 IU/ml in two consecutive measurements at least 3 months apart after cessation of treatment. These patients were all retreated with NUCs.

All patients were HIV seronegative and were not co-infected with hepatitis C or hepatitis delta virus.

For comparison, 8 HLA-A2 or HLA-B7-positive healthy controls were included. These were all seropositive for cytomegalovirus (CMV), which enabled us to compare characteristics of HBV-specific T cells (chronic infection with detectable viral load) with CMV-specific T cells (latent infection with undetectable viral load).

Viral assessments

Quantification of plasma HBV DNA, HBsAg and HBV genotyping was assessed as described previously [17]. CMV serostatus was determined by anti-CMV IgG in serum using the AxSYM microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL) according to the manufacturer's instructions.

Peripheral blood mononuclear cells (PBMCs)

Heparinized peripheral blood samples were obtained at baseline (all patients), and for CHB patients with HVL subsequently during treatment (week 4, 12 and 48), after treatment (week 52 and week 72) and at long-term follow-up (LFU) (range 144–196 weeks). PBMCs were isolated using standard density gradient centrifugation and subsequently cryopreserved until the day of analysis.

Immunofluorescent staining and flow cytometry

PBMCs were washed in PBA (PBS containing 0.01% (w/v) NaN_3, 0.5% (w/v) bovine serum albumin and 2 mM EDTA). Thawed PBMCs (1.0×10^6 cells) were incubated for 30 min in the dark at 4 °C with different combinations of fluorescent label-conjugated mouse antibodies. For phenotypic analysis, the following mAbs were used: CD45RA FITC, CD38 PE, CD161 PE, HLA-DR PerCP-Cy5.5, CD8 PerCP-Cy5.5, CD45RA PE-Cy7, CCR7 PE-Cy7 (BD Biosciences, San Jose, USA), CX3CR1 PE (MBL International, Naka-ku Nagoya, Japan), CXCR3 PE (R&D Systems, Minneapolis, USA), CD3 PE-Alexa610, CD27 APC-Alexa750 (Invitrogen, Camarillo, USA), CD127 PerCP-Cy5.5, CD8 Alexa700, PD-1 PE (eBioscience, San Diego, USA). For intracellular staining, cells were fixed after surface staining with FACS Lysing Solution (BD) and subsequently permeabilized (FACS Permeabilizing Solution 2 (BD)). Cells were incubated for 30 min in the dark at 4 °C with one or more of the following antibodies: perforin FITC, Ki67 FITC (BD Biosciences), granzyme B PE (Sanquin, Amsterdam, The Netherlands), granzyme K FITC (Immunotools, Germany) or T-bet PerCP-Cy5.5 (eBioscience, San Diego, USA). Measurements were done using BD FACS Canto or LSR Fortessa flow cytometer (BD Biosciences, Europe) and FACS Diva Software. Analysis was done using FlowJo MacV8.6.3.

Table 1. Baseline characteristics of subjects with chronic hepatitis B and healthy controls.

	HVL	HVL	HVL	LVL	Healthy controls (HC)
Treatment	PegIFN + ADV	PegIFN + ADV	PegIFN + ADV	n.a.	n.a.
Response	HBsAg loss	Combined response (CR)	Non-response (NR)	n.a.	n.a.
Number of subjects	7	7	14	22	8
Gender, male (%)	7 (100)	4 (57)	12 (86)	13 (59)	4 (50)
Age, median (range)	47 (38-49)	42 (25-62)	42 (27-53)	46 (20-65)	32 (29-56)
ALT U/L, median (range)	80 (26-275)	108 (26-199)	98 (22-1256)	26 (19-152)	n.a.
HBV DNA, log_{10} IU/ml, mean ± SD	6.45 ± 2.30	5.64 ± 2.14	7.10 ± 1.72	2.52 ± 1.15	n.a.
HBeAg status, pos (%)	3 (42)	2 (28)	7 (50)	0 (0)	n.a.
Viral genotype					n.a.
A	4	2	8	7	
B					
C					
D	1	3	4	7	
E	2	2	3	3	
non measurable				5	