Phenotype and function of monocyte derived dendritic cells in chronic hepatitis B virus infection

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The antiviral T cell failure of patients with chronic hepatitis B virus (HBV) infection was suggested to be caused by a T cell stimulation defect of dendritic cells (DC). To address this hypothesis, monocyte derived DC (MDDC) of patients with chronic or resolved acute HBV infection and healthy controls were studied phenotypically by FACS analyses and functionally by mixed lymphocyte reaction, ELISA, ELISpot and proliferation assays of MDDC cultures or co-cultures with an allogeneic HBc-specific Th cell clone. HBV infection of MDDC was studied by quantitative PCR. MDDC from HBV patients seemed to be infected by the HBV, showed a reduced surface expression of HLA DR and CD40 and exhibited a reduced secretion of IL12p70 in response to HBcAg but not to LPS, as compared to control MDDC. However, after cytokine induced maturation, MDDC from HBV patients revealed an unimpaired phenotype. Moreover, the T cell stimulatory capacity of HBV-DC was intact, since (i) the induction of allospecific proliferative and IFN-γ responses was not affected in HBV-MDDC, and (ii) HLA DR7 restricted stimulation of an allogeneic HBc-specific Th cell clone was not impaired by HBV-MDDC compared to control MDDC. It is hypothesized that HBV infection of DC might lead to minor phenotypic and functional alterations without significantly affecting their antiviral Th cell stimulatory capacity.

INTRODUCTION

Spontaneous recovery from acute HBV infection is believed to be mediated by strong antiviral immune responses, while approximately 10% of infected patients develop a chronic course with persistent DNA replication probably due to weak antiviral immune responses. Because dendritic cells (DC) are needed to prime B cell, cytotoxic T lymphocytes (CTL) and Th cell responses in vivo (Banchereau & Steinman, 1998) and might represent an extrahepatic reservoir of the virus (Lew & Michalak, 2001; Stoll-Becker et al., 1997), an impaired function of DC was suggested to account for the T and B cell hyporesponsiveness in chronic HBV infection by recent studies demonstrating the HBV infection of monocyte derived DC (MDDC) and a reduced expression of costimulatory molecules leading to impaired T cell allostimulation (Beckebaum et al., 2002; Kunitani et al., 2002; Wang et al., 2001).

The aim of our study was to assess the functional consequences of this MDDC phenotype in patients with various courses of chronic hepatitis B. Because isolation of DC from peripheral blood is hampered by the very low numbers and immature differentiation stage of circulating DC precursors (Banchereau & Steinman, 1998), a well established protocol to generate DC in vitro from monocytes by culture with IL4 and GM-CSF (Palucka et al., 1998; Romani et al., 1994) has been employed to study phenotype and function of MDDC from patients with chronic hepatitis B, healthy controls and donors with spontaneously resolved acute HBV infection (RHB).

METHODS

Patients. In total, 35 patients with chronic HBV infection were studied (Table 1): 18 had chronic active hepatitis B [CAH; elevated alanine aminotransferase (ALT) for longer than 6 months, seropositivity for HBsAg, HBeAg and anti-HBc Abs and positive HBV-DNA by hybridization assay or quantitative PCR (HBV-Amplicor; Roche Diagnostics)]; 17 were inactive HBs carriers [ISC; normal ALT (<42 U l⁻¹), seropositivity for anti-HBe and negative DNA (hybridization assay, cut off <2 pg ml⁻¹; PCR, detection limit 1 x 10⁶ viral genome equivalents (VGE) ml⁻¹)]. No patient under treatment had received antiviral treatment before or during the study.
had serious concomitant diseases. As controls, 22 healthy HBV-naive blood donors (seronegative for HBsAg, anti-HBs and anti-Hbc) and 6 healthy individuals with RHB (normal ALT, positive for anti-HBc and anti-HBs and negative for HBV-DNA) were assessed. All patients and controls tested seronegative for anti-HIV-1/2 and anti-HCV and gave informed consent according to the ethical guidelines from Helsinki. The study was approved by the institutional ethical committee.

**PBMC and CD4+ cell isolation, and HBC-specific Th cell clone.** PBMC were isolated from peripheral blood by Ficoll-density gradient centrifugation. CD4+ cells were separated from PBMC by the use of immunomagnetic anti-CD4-beads according to the manufacturer’s instructions (Milteniy Biotech). The HBCAg-specific and HLA DR7-restricted Th cell clone had been generated from a patient during the acute phase of a self-limited HBV infection, as described (Jung et al., 1995). After thawing, the clone was restimulated with allogeneic irradiated (3000 rad) PBMC and expanded in RPMI 1640 plus 5% human AB serum and IL2 (50 U/ml) for a further 3 weeks. Then, IL2 supplement was stopped and cells were used for co-incubation with allogeneic HLA DR7 matched MDDC 8 days later.

**MDDC culture.** Donor PBMC were incubated at 12 × 10^6 cells per well in 6-well plates for 60 min in PBS. After removal of non-adherent cells, the remaining cells were incubated in serum-free MDDC medium [X-Vivo 15 (Biowhittaker) supplemented with 800 U/ml GM-CSF (Leukomax) and 1000 U/ml IL4 (Strathmann Biotech)] at 37 °C. Cells were fed every 2 days with fresh medium. After 7 days, non-adherent immature MDDC were harvested. In maturation experiments, MDDC were cultured for an additional 2 days in X-Vivo 15 medium supplemented with IL1β, TNF-α (both: 10 ng/ml), R&D Systems; each: 1000 U ml⁻¹), IL6 (Strathmann Biotech: 1000 U ml⁻¹) and Prostaglandin (Pg) E2 (Sigma; 1 mg ml⁻¹).

**Flow cytometry of surface markers.** Expression of surface markers was analysed by flow cytometry using conjugated monoclonal mouse-anti-human antibodies: FITC-anti-CD1a, FITC-anti-CD3, FITC-anti-CD40, FITC-anti-CD83, PE-anti-CD14, PE-anti-CD86, PE-anti-HLA DR and CY-Chrome-anti-CD19 (Pharmingen). Analysis was performed on a FACScan (Becton Dickinson) utilizing the CellQuest software. Quadrants were set according to staining with the respective isotype controls.

**T cell stimulation (ELISPot, proliferation assay).** MDDC from patients and controls were harvested at day 7, before assessment by enzyme-linked immunospot assays (ELISPot) as described (Bocher et al., 1999). PVDF-membrane-bottomed 96-well plates (Millipore) were coated with 10 μg per well of mAb against IFN-γ (MabTech) in carbonate coating buffer. Patient and control MDDC were seeded in triplicates (5 × 10^5 per well) together with 1 × 10^6 per well CD4+ Th cells. Phytohaemagglutinin-stimulated MDDC/T cell co-cultures served as positive controls. MDDC were irradiated (3000 rad) and washed twice before use. After incubation for 40 h, cells were discarded and the plates were washed in PBS-0.05% Tween and incubated with biotinylated anti-IFN-γ mAb (1:1000; MabTech). After washing, plates were incubated with HRP-Extravidin, washed again and incubated with AEC solution (both Sigma). The staining was stopped by rinsing under water and red spots were counted as single spot forming cells (SFC). Frequencies of allogeneic T cells were calculated from the SFC in immature MDDC/T cell co-cultures corrected for the background SFC of MDDC monocultures.

**Mixed lymphocyte reactions (MLR).** For stimulation of the HBC-specific Th cell clone, PBMC from 4 HLA DR7-positive chronic HBV patients and healthy controls each was stored frozen and thawed simultaneously, cultured for 7 days and pulsed with medium or HBC antigen as described above. A mononuclear anti-DR antibody (10 μg/ml; Pharmingen) was added to analyse HLA restriction. MDDC (5 × 10^4 per well) and clonal T cells (1 × 10^5 per well) were co-incubated in triplicates in 96-well plates for 4 days and pulsed with 0.25 μCi (9.25 kBq) per well [3H]thymidine and read by liquid scintillation technique. The results were expressed as antigen specific stimulation index (SI) calculated as the ratio of stimulated and unstimulated cultures. Baseline counts were similar for patient and control cultures (range: 215–700 c.p.m. and 180–560 c.p.m., respectively).

**Cytokine secretion by MDDC.** Supernatants of MDDC were stimulated or not for 48 h with varying doses of HBCAg (4 and 20 μg ml⁻¹; Diasorin) or LPS (0-1, 1, 10 μg ml⁻¹; Sigma) used to assess the secretion of TNF-α, IL10, and IL12p70 by ELISA according to the manufacturer’s instructions (OptEIA; Pharmingen). Background values of unstimulated MDDC cultures were between 10 and 60 pg ml⁻¹ and 0 and 50 pg ml⁻¹ for IL12 and IL10, respectively, and did not differ between donor groups.

**HBV-DNA and antigen expression.** CD14+ monocytes, CD19+ B cells and CD4+ or CD8+ T cells were separated from PBMC using immunomagnetic beads for the respective surface marker (Miltenyi Biotech). In some experiments, MDDC were enriched further by immunomagnetic depletion of CD14+, CD19+ and CD8+ cells. Cellular DNA was isolated from 1 to 2.5 × 10^6 cells by the Qiagen method (Qiagen), resuspended in a total volume of 100 μl distilled water and the DNA content was quantified.

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (years)</th>
<th>HBsAg</th>
<th>HBeAg</th>
<th>Anti-HBc</th>
<th>ALT+ (U l⁻¹)</th>
<th>HBV-DNA+ (pg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAH (n=18)</td>
<td>39±16</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>204±265</td>
<td>2763±2630</td>
</tr>
<tr>
<td>ISC (n=17)</td>
<td>39±11</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>35±13</td>
<td>0±6±2.4</td>
</tr>
<tr>
<td>RHB (n=6)</td>
<td>40±12</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>23±11</td>
<td>0±0</td>
</tr>
<tr>
<td>Controls (n=22)</td>
<td>34±8</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

*Normal range <41 U l⁻¹.
†Determined by dot blot hybridization assay.

**Table 1. Clinical and serological patient data**

Values expressed as mean±SD; ALT, alanine aminotransferase; +/−, positive/negative; NT, not tested.
photometrically. Cellular and serum HBV-DNA was detected quantitatively using the HBV-Amplicor assay according to manufacturer’s instructions (Roche Diagnostics).

For expression studies of HBs antigen, the pellet of at least 2 × 10⁶ MDDC was lysed in lysis buffer of 1% Triton X, 1 mM PMSF (Roche Diagnostics; #1697498), 50 mM Tris in 150 mM NaCl (pH 8-0) and stored at −20°C until analysis in a commercial HBsAg ELISA according to manufacturer’s instructions (ETI-MAK-3; Diasorin Biomedica).

Statistics. The Mann-Whitney test for non-parametric data were calculated utilizing the StatView software (SAS Institute). P-values <0.05 were regarded as significant. The MLR proliferation data were analysed as summary measures between patients and controls using the student t-test for impaired data.

RESULTS

Phenotype of MDDC from chronic HBV patients and controls

When studied by FACScan after 7 days of culture, MDDC derived from HBV-naive controls revealed a strong expression of the surface markers CD1a (34 ± 17% positive cells), CD40, CD86 and HLA DR (Table 2, Fig. 1), whereas the expression of CD1a (CAH: 20 ± 10%, ISC: 11 ± 7% positive cells), CD40, HLA DR and CD86 on MDDC generated from ISC and CAH patients was widely reduced without significant differences between CAH and ISC patients. The surface expression of lineage markers (CD3, CD19 and CD14) and the DC differentiation marker CD83 was below 5% in all cultures. Interestingly, the phenotype of MDDC from RHB donors also showed reduced expression of HLA DR and CD86, while CD40 was significantly higher than on MDDC from controls and patients (Fig. 1, Table 2).

In situ, after encounter of antigen or proinflammatory cytokines, DC undergo further maturation while migrating to regional lymphoid tissues, where T cells are stimulated by mature DC. In order to mimic these physiological conditions, MDDC from HBV patients and controls were cultured for 2 days in the presence of a proinflammatory cytokine cocktail (IL1β, IL6, TNF-α and PgE2), mimicking DC maturation by CD40 ligation (Jonuleit et al., 1997). Indeed, exposure to proinflammatory cytokines led to upregulation of CD40, CD86, CD83 and HLA DR and downregulation of CD1a on MDDC of patients and controls resulting in equivalent expression rates and mean fluorescence intensities on mature MDDC of all groups (Table 3).

Cytokine secretion by MDDC

Because a reduced secretion of IL10 or IL12 by DC could cause reduced Th cell responses, supernatants of MDDC were analysed after 48 h of stimulation with various doses of LPS or HBCAg. Interestingly, in response to HBCAg both IL12p70 and IL10 were found to be reduced quantitatively in MDDC cultures from CAH and ISC patients compared with healthy controls or RHB donors (Fig. 2). However, LPS stimulated MDDC cultures demonstrated a slightly reduced IL10 secretion by ISC (P <0.09) but otherwise unimpaired cytokine secretion. No differences were found regarding TNF-α secretion (not shown).

HBV gene and antigen expression in patient MDDC

Because these phenotypic and functional alterations of MDDC from chronic HBV patients might result from interaction of HBV genes with the host cell, a quantitative PCR assay was used to study HBV-DNA expression in monocytes and MDDC from patients and controls. Indeed, high numbers of VGE were detected in MDDC from CAH patients, while MDDC of ISC were negative or had significantly lower copy numbers and MDDC of all but one RHB donors were negative (Table 4). In one RHB donor with low-level persisting viraemia, HBV-DNA also could be detected in the MDDC.

When MDDC were enriched further by immunomagnetic depletion of the low numbers of contaminating CD14+CD19+ and CD8+ cells, HBV-DNA was detected exclusively in the MDDC fraction but not in the depleted lineage-marker positive cell population (not shown).

The transcription of HBV genes in MDDC from a total of nine CAH and ISC patients was analysed by ELISA and uncovered the expression of HBsAg in MDDC from two

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Table 2. Expression of costimulatory molecules on DC from different patient groups indicated as percentage of positive cells of the ungated cell population

<table>
<thead>
<tr>
<th>Patient group</th>
<th>CD86</th>
<th></th>
<th>HLA DR</th>
<th></th>
<th>CD40</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% pos. cells</td>
<td>P-value*</td>
<td>% pos. cells</td>
<td>P-value*</td>
<td>% pos. cells</td>
</tr>
<tr>
<td>CAH (n=10)</td>
<td>60 ± 19</td>
<td>P &lt;0.05</td>
<td>43 ± 25</td>
<td>P &lt;0.01</td>
<td>23 ± 21</td>
</tr>
<tr>
<td>ISC (n=9)</td>
<td>66 ± 18</td>
<td>NS</td>
<td>53 ± 35</td>
<td>P &lt;0.05</td>
<td>21 ± 24</td>
</tr>
<tr>
<td>CTR (n=22)</td>
<td>72 ± 14</td>
<td>-</td>
<td>76 ± 16</td>
<td>-</td>
<td>44 ± 30</td>
</tr>
<tr>
<td>RHB (n=4)</td>
<td>58 ± 3</td>
<td>NS</td>
<td>54 ± 9</td>
<td>P &lt;0.05</td>
<td>91 ± 7</td>
</tr>
</tbody>
</table>

*P-value of % positive cells compared with healthy CTR.
CAH patients, both with high serum DNA levels ($2 \times 10^7$ and $1.2 \times 10^{10}$ VGE ml$^{-1}$), while MDDC from a further five CAH and one ISC patients were HBsAg-negative, probably due to low-level viral transcription or the low amount of protein available from the MDDC lysate.

**Alloreactive Th cell stimulation**

In previous studies, MLR have been used to study the T cell stimulatory capacity of MDDC from HBV patients. Because allostimulation depends on the degree of HLA mismatch

**Table 3.** Expression of costimulatory molecules on DC from different patient groups before and after induction of maturation by proinflammatory cytokines, calculated as MFI and percentage positive cells (mean ± SD)

<table>
<thead>
<tr>
<th>Patient group</th>
<th>CD 86</th>
<th>HLA DR</th>
<th>CD 40</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immature</td>
<td>Mature</td>
<td>Immature</td>
</tr>
<tr>
<td></td>
<td>MFI</td>
<td>% pos.</td>
<td>MFI</td>
</tr>
<tr>
<td>CAH</td>
<td>128 ± 79</td>
<td>60 ± 19</td>
<td>570 ± 508</td>
</tr>
<tr>
<td>ISC</td>
<td>65 ± 41</td>
<td>66 ± 18</td>
<td>509 ± 82</td>
</tr>
<tr>
<td>RHB</td>
<td>39 ± 16</td>
<td>41 ± 30</td>
<td>110 ± 89</td>
</tr>
<tr>
<td>CTR</td>
<td>108 ± 80</td>
<td>82 ± 12</td>
<td>248 ± 54</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with CTR.
†P < 0.05 compared with RHB.
between stimulator and responder cells, immature MDDC and responder T cells from a rather large number of donors were assessed in our study: MDDC derived from six healthy individuals or six HBV patients were co-incubated with a panel of separated CD4+ Th cells or PBMC derived from five allogeneic healthy donors and the frequencies of alloreactive T cells were analysed by IFN-γ-ELISpot or proliferation assay. In contrast to the previous studies, both sets of experiments did not reveal significant differences between patient and control MDDC in stimulating alloreactive IFN-γ secretion (Fig. 3a) or proliferation (P > 0.05; Fig. 3b), arguing strongly against a reduced T cell stimulation by patient DC.

**Antigen-specific Th cell stimulation**

The above finding of uncompromised allospecific T cell stimulation does not exclude the failure of patient DC to stimulate antigen-specific T cell responses, because MLR does not assess a number of important APC functions (i.e., antigen uptake and processing). However, antigen-specific T cell stimulation requires HLA restricted antigen presentation necessitating an autologous or HLA class II matched experimental setting. Because autologous MDDC/T cell co-cultures would not allow differentiation between a stimulator or responder cell defect, an allogeneic co-culture setting was established using an HBc-specific HLA DR7-restricted Th cell clone from a patient with acute self-limited hepatitis B (Jung et al., 1995). This responder Th cell clone was co-incubated with allogeneic HLA DR7 matched MDDC from four chronic HBV patients and healthy controls and specific T cell stimulation was assessed by proliferation assays and cytokine ELISA. The whole experiment was performed simultaneously with MDDC generated from frozen PBMC thawed, cultured and harvested at the same time from all patients and controls, in order to avoid artefacts. The results demonstrated equivalent clonal T cell proliferation and IFN-γ secretion after stimulation by patient or control MDDC, while IL10 secretion was low in all cultures, corresponding to the well known Th1 type of this clone (Fig. 4). The T cell stimulation was specific and HLA DR mediated since a monoclonal anti-DR antibody widely inhibited T cell activation in all cultures (not shown).

**DISCUSSION**

Weak and oligospecific antiviral B and T cell responses are believed to be responsible for the lack of virus control in chronic HBV infection. However, the mechanisms underlying this immunological hyporesponsiveness are still unknown (Bertoletti & Ferrari, 2003; Chisari & Ferrari, 1995). In various viral diseases, such as vaccinia virus infection (Engelmayr et al., 1999), measles virus infection (Servet-Delprat et al., 2000) or chronic hepatitis C virus (HCV) infection (Auffermann-Gretzinger et al., 2001; Bain et al., 2001; Kanto et al., 1999), the infection of DC is associated with impaired DC differentiation and function. Thus, infection of DC and interaction with their APC function by downregulation of costimulatory molecules may represent a rather common mechanism by which viruses establish persistent infection. In previous studies, a functional defect of DC in chronic HBV infection was suggested, that might cause a reduction of antiviral Th cell

**Table 4.** HBV-DNA expression in DC and CD8 cells from ISC, CAH patients and RHB donors

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>HBe</th>
<th>ALT</th>
<th>Serum*</th>
<th>DC†</th>
<th>CDB†</th>
</tr>
</thead>
<tbody>
<tr>
<td>W. W.</td>
<td>CAH</td>
<td>+</td>
<td>105</td>
<td>3 × 10^3</td>
<td>750</td>
<td>0</td>
</tr>
<tr>
<td>S. A.</td>
<td>CAH</td>
<td>+</td>
<td>621</td>
<td>3 × 10^6</td>
<td>2875</td>
<td>NT</td>
</tr>
<tr>
<td>S. M.</td>
<td>CAH</td>
<td>+</td>
<td>135</td>
<td>3 × 10^5</td>
<td>19230</td>
<td>NT</td>
</tr>
<tr>
<td>D. T.</td>
<td>CAH</td>
<td>+</td>
<td>696</td>
<td>4 × 10^3</td>
<td>114286</td>
<td>0</td>
</tr>
<tr>
<td>S. J.</td>
<td>CAH</td>
<td>+</td>
<td>44</td>
<td>2-9 × 10^3</td>
<td>105</td>
<td>NT</td>
</tr>
<tr>
<td>D. K.</td>
<td>CAH</td>
<td>−‡</td>
<td>79</td>
<td>1-8 × 10^5</td>
<td>608</td>
<td>NT</td>
</tr>
<tr>
<td>M. G.</td>
<td>ISC</td>
<td>−</td>
<td>19</td>
<td>0</td>
<td>250</td>
<td>0</td>
</tr>
<tr>
<td>S. A.</td>
<td>ISC</td>
<td>−</td>
<td>18</td>
<td>0</td>
<td>140</td>
<td>0</td>
</tr>
<tr>
<td>F. B.</td>
<td>ISC + NASH</td>
<td>−</td>
<td>80</td>
<td>0</td>
<td>125</td>
<td>NT</td>
</tr>
<tr>
<td>A. R.</td>
<td>ISC</td>
<td>−</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D. S.</td>
<td>ISC</td>
<td>−</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>W. K.</td>
<td>ISC</td>
<td>−</td>
<td>23</td>
<td>0</td>
<td>180</td>
<td>NT</td>
</tr>
<tr>
<td>S. M.</td>
<td>RHB</td>
<td>−</td>
<td>51</td>
<td>0</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>M. A.</td>
<td>RHB</td>
<td>−</td>
<td>22</td>
<td>1 × 10^3</td>
<td>12200</td>
<td>NT</td>
</tr>
<tr>
<td>O. E.</td>
<td>RHB</td>
<td>−</td>
<td>29</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R. W.</td>
<td>RHB</td>
<td>−</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>NT</td>
</tr>
</tbody>
</table>

*VGE ml⁻¹ serum by PCR based Amplicor assay (detection limit 1 × 10⁵ VGE ml⁻¹).
†VGE μg⁻¹ isolated DNA by PCR based Amplicor assay.
‡Suspected infection by preCore stop mutant virus.
§Histologically proven non-alcoholic steatohepatitis.
and CTL reactivities (Beckebaum et al., 2002; Löhr et al., 2002; Wang et al., 2001). However, uncommon protocols to generate DC from stem cells or monocytes, heterogeneous patient populations and limited functional experiments make the interpretation of these studies difficult. Thus, we aimed to extend these studies utilizing a well established and widely used in vitro culture system to generate DC from monocytic precursors (Palucka et al., 1998; Romani et al., 1994), thereby mimicking an important pathway of DC differentiation in vitro (Randolph et al., 1998) and in vivo (Randolph et al., 1999).

The central mechanisms of DC-mediated T cell stimulation after antigen uptake and processing by the APC comprise the engagement of the antigen-specific T cell receptor by peptide loaded MHC molecules, interaction of costimulatory molecules with their receptors on the T cell and the secretion of cytokines. Our FACS analyses revealed a significantly reduced expression of CD40 and HLA DR and a slight reduction of CD86 on MDDC from ISC and CAH patients. This rather immature phenotype is in concordance with Wang et al. (2001), whereas Beckebaum et al. (2002) using a different protocol to generate MDDC found only the expression of CD80 and CD86 reduced. In our study, a reduced expression of CD86 and HLA DR was detected even years after resolution from self-limited HBV infection, while CD40 was strongly induced on RHB-MDDC indicating a permanent influence of the virus on MDDC phenotype. However, the reduced expression of costimulatory molecules of immature patient MDDC was reversed after cytokine induced maturation and was not associated with reduced allostimulation of healthy Th cells in IFN-γ secretion and proliferation assays, indicating the lack of functional relevance of this finding. Although this is in contrast to previous reports of reduced allostimulation by DC from HBV patients (Beckebaum et al., 2002; Wang et al., 2001), different protocols to generate MDDC, heterogeneous patient populations and the examination of only one or two healthy responder cell donors are important limitations of these reports. Moreover, a reduced allostimulation by DC would indicate a general T cell stimulation failure of chronic HBV patients that would contrast to the clinical immune competence of chronic HBV patients.

The key cytokines provided by APC determining the induced Th cell response towards a Th1 or Th2 cell cytokine pattern are TNF-α, IL10 and IL12 (Trinchieri & Scott, 1994) that are secreted by DC in response to various viral, protozoal or bacterial products (Ahuja et al., 1998; Hilkens et al., 1997; Macatonia et al., 1995; von Stebut et al., 1998). In Hbc-antigen stimulated MDDC cultures, MDDC derived from CAH or ISC patients indeed showed a reduced secretion of IL12 and IL10 compared with RHB and HBV-naive controls, corresponding to previous findings under different stimulation conditions (Beckebaum et al., 2002; Wang et al., 2001). In contrast, after LPS stimulation the secretion of both cytokines was intact, precluding a general cytokine secretion defect.

**Fig. 3.** MLR of healthy Th cells stimulated by patient or control MDDC. (a) IFN-γ secretion of alloreactive CD4 cells derived from five healthy donors (CD4-1, -5) and stimulated by MDDC from six HBV patients (HBV-DC) or six HBV-naive controls (CTR-DC). Frequencies of alloreactive IFN-γ spot forming cells (SFC) were detected after incubation of MDDC and allogeneic CD4 cells (ratio 1:2) for 40 h by ELISpot. (b) Proliferation of alloreactive PBMC from an HBV-naive donor stimulated for 4 days by MDDC from six HBV patients (HBV-DC) or six HBV-naive controls (CTR-DC). This experiment is representative for five experiments performed with responder PBMC from five healthy donors.

**Fig. 4.** Hbc antigen-specific stimulation of an HLA DR7 restricted Th cell clone by MDDC from four chronic HBV patients and healthy HBV-naive controls each. (a) Hbc-specific T cell proliferation is presented as stimulation index (SI: ratio of c.p.m. in Hbc antigen stimulated versus unstimulated cultures). (b) The cytokines were determined in 24 h supernatants of the co-cultures by ELISA.
In order to assess, whether this HBc-specific cytokine secretion failure might have functional implications on HBc-specific T cell stimulation, the response of an HBc-specific Th cell clone to stimulation by HLA DR matched MDDC from HBV patients and controls was studied. The finding of identical proliferation and IFN-γ secretion by the Th1 cell clone in co-cultures with MDDC from chronic HBV patients or controls ruled out a significant stimulation failure of patient MDDC and rather argues for a virus-specific responder Th cell defect.

HBV might cause these phenotypic and functional alterations by direct infection of DC precursors in the blood or bone marrow, as suggested by our PCR and ELISA studies and the report of Beckebaum et al. (2002), demonstrating the expression of HBV-DNA, particles and antigens in MDDC from HBV patients. However, whether DC are a site of HBV replication or gene expression is not clear yet, but from our studies the proof of HBV in MDDC seems to be irrelevant for the function of DC, particularly in the light of an otherwise fully competent immune system of chronic HBV patients.

Finally, our data, just as those of previous studies, have to be interpreted in consideration of using an in vitro model to generate DC from monocyctic precursors. Although this differentiation pathway has been reported in vitro and in vivo (Randolph et al., 1998, 1999), artefacts cannot be excluded because the resulting cell population might contain different DC and non-DC populations. Thus, ex vivo studies on purified DC are under way to assess whether these data are applicable to DC subpopulations in vivo.

In conclusion, our data suggest the infection of myeloid DC by the HBV leading to minor phenotypic and functional alterations with reduced expression of costimulatory molecules and IL10/IL12 secretion under certain experimental conditions. However, the Th cell stimulatory capacity of MDDC from chronically-infected HBV patients was unaffected by these changes, indicating that mechanisms other than a DC defect led to the well described antiviral T cell failure in chronic hepatitis B.

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