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Strong and multi-antigen specific immunity by hepatitis B core antigen (HBcAg)-based vaccines in a murine model of chronic hepatitis B: HBcAg is a candidate for a therapeutic vaccine against hepatitis B virus

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ABSTRACT

Experimental evidence suggests that hepatitis B core antigen (HBcAg)-specific cytotoxic T lymphocytes (CTL) are essential for the control of hepatitis B virus (HBV) replication and prevention of liver damage in patients with chronic hepatitis B (CHB). However, most immune therapeutic approaches in CHB patients have been accomplished with hepatitis B surface antigen (HBsAg)-based prophylactic vaccines with unsatisfactory clinical outcomes. In this study, we prepared HBsAg-pulsed dendritic cells (DC) and HBcAg-pulsed DC by culturing spleen DC from HBV transgenic mice (HBV TM) and evaluated the immunomodulatory capabilities of these antigens, which may serve as a better therapy for CHB. The kinetics of HBsAg, antibody levels against HBsAg (anti-HBs), proliferation of HBsAg- and HBcAg-specific lymphocytes, production of antigen-specific CTL, and activation of endogenous DC were compared between HBV TM vaccinated with either HBsAg- or HBcAg-pulsed DC. Vaccination with HBsAg-pulsed DC induced HBsAg-specific immunity, but failed to induce HBcAg-specific immunity in HBV TM. However, immunization of HBV TM with HBcAg-pulsed DC resulted in: (1) HBsAg negativity, (2) production of anti-HBs, and (3) development of HBsAg- and HBcAg-specific T cells and CTL in the spleen and the liver. Additionally, significantly higher levels of activated endogenous DC were detected in HBV TM immunized with HBcAg-pulsed DC compared to HBsAg-pulsed DC ($p < 0.05$). The capacity of HBcAg to modulate both HBsAg- and HBcAg-specific immunity in HBV TM, and activation of endogenous DC in HBV TM without inducing liver damage suggests that HBcAg should be an integral component of the therapeutic vaccine against CHB.

1. Introduction

Despite the considerable information regarding the viral life cycle, epidemiology, immunology, pathogenesis and prevention of hepatitis B virus (HBV), there has been a lack of significant developments in treating patients with chronic hepatitis B (CHB). Several antiviral drugs have been developed for treating CHB patients during the last three decades. However, controversy remains about their therapeutic efficacy. A systemic review of the National Institutes of Health (NIH) Consensus Development Conference, which assessed all randomized clinical trials on antiviral drugs in CHB patients from 1989 to 2008, revealed that antiviral drug treatment did not improve the clinical outcomes and all intermediate outcomes in CHB patients in any credible randomized-controlled trial (Shamliyan et al., 2009; Wilt et al., 2008). However, others have shown that these drugs could block or delay the progression of liver disease in CHB patients (Liaw, 2009; Lin et al., 1999). Although it is difficult to determine the underlying causes of these discrepancies, as different investigators used different criteria in their therapeutic evaluations, it is generally accepted that an ongoing treatment regimen for CHB with antiviral drugs is not satisfactory, and has low efficacy and considerable adverse effects. In addition, it is now clear that antiviral drugs possess poor immunomodulatory capabilities, which may be responsible for their ineffective control of HBV replication and inadequate prevention of liver damage in CHB (Lok and McMahon, 2007; Liaw and Chu, 2009).

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Clinical and experimental evidence suggests that the replication of HBV DNA and progression of liver damage is under control in many CHB patients, even those not receiving any antiviral drug therapy. The magnitude and nature of host immunity to HBV is important in regulating these pathological events in CHB. In support of this concept, Maini et al. (2000) demonstrated that CHB patients that are capable of controlling HBV replication and liver damage harbor higher frequencies of hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg)-specific cytotoxic T lymphocytes (CTL) compared to those that express high levels of HBV and have progressive liver damage. Taken together, it appears that the restoration of host immune responses to HBV-related antigens may have therapeutic implications in CHB patients.

Based on these observations, polyclonal immunomodulators, such as cytokines, growth factors, and other immune mediators, were used in CHB patients. However, they had limited therapeutic efficacy and considerable side effects in CHB patients (Sprengers and Janssen, 2005). Subsequently, an antigen-specific immunotherapeutic approach, or vaccine therapy, was developed for CHB patients, which used commercially available prophylactic hepatitis B (HB) vaccines for treating CHB patients. Different investigators used different types of vaccines with different immunization protocols, and thus, it is difficult to assess the real therapeutic implications of vaccine therapy in CHB patients (Wang et al., 2010; Hoa et al., 2009; Pol et al., 2001). Indeed, it appears that the HBsAg-based vaccine may not be an effective immunotherapeutic approach in CHB. A well-planned clinical trial in 80 patients with CHB used a HBsAg-based vaccine in combination with another antiviral drug also failed to exhibit substantial therapeutic effect (Vandepapelière et al., 2007). Conversely, Heathcote et al. (1999) used a HBcAg epitope-based vaccine in CHB patients and achieved moderate therapeutic effects. Recently, Luo et al. (2010) reported that antigen-pulsed dendritic cells (DC) containing epitope of HBsAg and HBcAg had therapeutic effects in hepatitis B e antigen (HBeAg)-negative patients, but not in HBeAg-positive patients.

These clinical trials with HBsAg- and HBcAg-based vaccines have raised more questions than solutions regarding immune therapy for CHB patients, as the mechanisms of action of HBsAg- or HBcAg-based vaccine in CHB have not yet been explored. However, most cellular and molecular events following vaccination with either HBsAg or HBcAg could not be evaluated in CHB patients due to ethics, safety, technical, and procedural limitations.

To develop proper insights about immunogenecity of HBsAg- or HBcAg-based therapeutic vaccines in CHB, the role of DC in adaptive immunity has been examined. DC, the most potent antigen-presenting cells, are responsible for processing and presenting antigens for induction of antigen-specific immune responses in normal conditions as well as in the immune tolerance state (Steinman and Banchereau, 2007). Studies have shown that the phenotypes and functions of DC are distorted in chronic HBV infections (van der Molen et al., 2004). One way to circumvent immune tolerance state is to produce antigen-pulsed DC and use them as a vaccine. In fact, cancer antigen-pulsed DC and HBsAg-pulsed DC have been used to induce cancer-specific immunity and HBsAg-specific immunity in cancer patients and CHB patients, respectively, when antigen-specific immune responses could not be properly induced by only cancer antigen or HBsAg (Banchereau and Palucka, 2005; Steinman and Banchereau, 2007; Akbar et al., 2010a).

The present preclinical study assessed the immunomodulatory mechanisms of HBsAg and HBcAg in a murine model of HBV, specifically HBV transgenic mice (TM). After immunizing HBV TM with antigen-pulsed DC, the immune responses of HBsAg-pulsed DC or HBcAg-pulsed DC were compared in the spleen and liver. This study may provide further insight into developing an immune therapy for CHB patients.

2. Methods

2.1. Mice

HBV TM (official designation, 1.2HB-BS10) were prepared by microinjecting the complete genome of HBV plus 619 bp of HBV DNA into the fertilized eggs of C57BL/6 mice. HBV TM are known to express HBV DNA and mRNAs of 3.5, 2.1, and 0.8 kbp of HBV in the liver (Araki et al., 1989). HBV DNA were also detected in the liver, and HBsAg was found in the sera of all HBV TM. Eight-week-old male C57BL/6 mice were purchased from Nihon Clea (Tokyo, Japan). Mice were housed in polycarbonate cages in our laboratory facilities, and maintained in a temperature- and humidity-controlled room (23 ± 1 °C) with a 12-h light/dark cycle. All mice received humane care, and the study protocol was approved by the Ethics Committee of the Graduate School of Medicine, Ehime University, Japan. Eight-week-old C3H/He mice (Nihon Clea) were used in an allogenic mixed leukocyte reaction (MLR).

2.2. Detection of HBV-related markers

HBsAg levels and antibodies against HBsAg (anti-HBs) in sera were estimated with a chemiluminescence enzyme immunoassay (Special Reference Laboratory, Tokyo, Japan) and expressed as IU/ml and mIU/ml, respectively, as previously described (Akbar et al., 2010b).

2.3. Isolation of T lymphocytes, B lymphocytes, and DC

We have previously described in detail the methodology for isolating spleen cells and liver nonparenchymal cells (NPCs) (Akbar et al., 2010b; Chen et al., 2011; Yoshida et al., 2010). To produce a single cell suspension from the spleen, spleens were cut into pieces and passed through a 40-μm-pore nylon filter (BD Falcon, Durham, NC, USA). The resulting cells were collected and suspended in culture medium containing RPMI 1640 (Iwaki, Osaka, Japan) with 10% fetal calf serum (Filtron PTY Ltd., Brooklyn, Australia).

To retrieve liver NPCs, liver tissues were cut into pieces, homogenized, passed through 70-μm-pore steel meshes (Morimoto Yakuhin Co., Matsuyama, Japan), and suspended in 35% percoll (Sigma Chemical, St. Louis, MO, USA). After centrifugation for 15 min at $450 \times g$ at room temperature, a high-density cell pellet was collected and suspended in culture medium.

T lymphocytes were isolated from the spleen single cell suspension or liver NPC by a negative selection column method using a mouse pan T isolation kit (Miltenyi Biotec, Bergish Gladbach, Germany), according to the manufacturer's directions (Chen et al., 2011; Yoshida et al., 2010).

DC were isolated from single cell suspensions of spleen and liver NPC using a density column (specific gravity 1.082), plastic adherence, re-culturing on plastic surface, and depletion of macrophages and lymphocytes or via positive selection of $CD11c^+$ cells with flow cytometry, as described (Akbar et al., 2010b, Chen et al., 2011).

2.4. Preparation of antigen-pulsed DC for immunizing HBV TM

HBsAg and HBcAg were purchased from Tokyo Institute of Immunology (Tokyo, Japan). Murine antigen-pulsed DC were prepared based on data from preliminary studies and according to our previous report (Akbar et al., 2010b; Miyake et al., 2010). Briefly, spleen DC were cultured with phosphate buffered solution (PBS) (unpulsed DC) or pyruvate dehydrogenase complex (PDC,

Sigma Biochemical, St. Louis, MO, USA) or HBsAg or HBcAg in culture medium for 48 h. DC were recovered from the cultures and washed five times with PBS. The viability of DC was assessed with a trypan blue exclusion test. The production of cytokines and T cell stimulatory capacities of antigen-pulsed DC were assessed *in vitro*.

2.5. Immunization schedule

HBV TM with comparable sera levels of HBsAg were used for this study. HBV TM were injected with either 5×10^6 unpulsed DC or 5×10^6 PDC-pulsed DC or 5×10^6 HBsAg-pulsed DC, or 5×10^6 HBcAg-pulsed DC. All vaccinations were done via the intraperitoneal route, six times, at an interval of 2 weeks. HBV TM were bled from the tail vein at different time intervals for assessments of various immunological parameters. HBV TM were sacrificed at different times after the initiation of immunization to estimate vaccine-induced cellular immune responses in the spleen and liver.

2.6. Lymphoproliferative assays

As described previously, murine lymphocytes were cultured in the absence or presence of different antigens to evaluate antigen-specific cellular immune responses (Akbar et al., 2010b; Chen et al., 2011; Yoshida et al., 2010). All cultures were performed in 96-well U-bottom plates (Corning Incorporated, New York, NY, USA). ^{3}H-thymidine (1.0 μCi/ml, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) was diluted in sterile PBS, added to the cultures for the last 16 h, and harvested automatically via a multiple cell harvester (LABO MASH, Futaba Medical, Osaka, Japan) onto filter paper (LM 101–10, Futaba Medical). [^{3}H]-thymidine levels of incorporation were determined with a liquid scintillation counter (Beckman LS 6500, Beckman Instruments, Inc., Fullerton, CA, USA) at blastogenesis. Triplicate cultures were assayed routinely and the results were expressed as counts per minute (cpm). The stimulation index was calculated as the ratio of cpm obtained in the presence of antigen or antigen-pulsed DC to that obtained without antigen or in presence of only DC or irrelevant antigen-pulsed DC (i.e., control culture). A stimulation index >3.0 was considered significant.

2.7. ELISPOT assay

$CD8^+$ T lymphocytes (1×10^5) were stimulated with the antigen in presence of mitomycin C-treated spleen adherent cells in an IFN-γ coated ELISPOT plate (Mabtech, Nacka Strand, Sweden) for 24 h (Akbar et al., 2010a; Yoshida et al., 2010). Subsequently, biotinylated antibodies (2A5-biotin, Mabtech) were added into the wells. After 2 h of incubation, the plates were incubated with streptavidin–alkaline phosphatase for 1 h. After washing the plates, the substrate solution, BCIP/NBT, was added. The reaction was stopped by washing the plates extensively with tap water. The numbers of spot-forming units (SFU) were counted using an ELISPOT reader (KS ELISPOT, Carl Zeiss, Thornwood, NY, USA), and subtracted from the numbers of background SFU of control wells.

2.8. Estimation of cytokine levels

Various cytokine levels were estimated in culture supernatants using a commercial kit for the cytometric bead array method, as previously described (Akbar et al., 2010b; Yoshida et al., 2010). Cytokines levels were calibrated to the mean fluorescence intensities of the standard negative control, standard positive control, and samples with Cytometric Bead Array software (BD Biosciences Pharmingen, San Jose, CA, USA) on a Macintosh computer (SAS Institute, Cary, NC, USA).

2.9. Statistical analysis

Data are shown as mean ± standard deviation (SD). Differences were compared using the Student's *t* test. For differences determined by the *F* test, the *t* test was adjusted for unequal variances (Mann–Whitney's *U*-test). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Evaluation of specificity of the experimental system

To assess the specificity of the experimental system, we immunized normal C57BL/6 mice twice with 10 μg of HBsAg or 10 μg of HBcAg, or 10 μg of PDC to induce antigen-specific lymphocytes in normal mice. Antigen-pulsed DC were prepared by culturing spleen DC from normal C57BL/6 mice with different antigens. HBsAg-, HBcAg-, and PDC-pulsed DC induced significant lymphoproliferation in mice immunized with HBsAg-, HBcAg-, and PDC, respectively. However, HBsAg-specific lymphoproliferation was not detected in mice immunized with HBcAg-pulsed DC or PDC-pulsed DC. Additionally, HBcAg and PDC-specific lymphocytes could not be retrieved from mice immunized with non-relevant antigens (Table 1). HBcAg-pulsed DC induced significantly higher levels of antigen-specific lymphoproliferation compared to HBsAg-pulsed DC (stimulation index, 31 ± 5 versus 15 ± 3.2, $N = 5$, $p < 0.05$) (Table 1).

After assessing the immunogenecity of antigen-pulsed DC of normal C57BL/6 mice *in vitro*, we prepared antigen-pulsed DC from HBV TM. Antigen-pulsed DC from HBV TM produced significantly higher levels of IFN-γ and IL-12 compared to unpulsed DC ($p < 0.05$). They also induced antigen-specific lymphoproliferation in normal mice immunized with the respective antigens (data not shown).

3.2. HBsAg negativity and anti-HBs production by antigen-pulsed DC

To assess if antigen-pulsed DC were capable of inducing HBsAg negativity and anti-HBs production in HBV TM, we checked HBsAg and anti-HBs in these mice at different times after vaccinations. HBsAg and anti-HBs levels were assessed in the sera of HBV TM before (0), and after 2, 4, and 6 vaccinations with various combinations of antigen-pulsed DC. All HBV TM expressed HBsAg in the sera, and anti-HBs were not detected in any of the mice prior to vaccination. In each group, 15 HBV TM were included for analyses.

Immunization of HBV TM with unpulsed DC or PDC-pulsed DC did not result in significant alteration in serum HBsAg levels.

Table 1
Antigen-specific proliferation of T cells by antigen-pulsed dendritic cells.

Lymphocytes	Dendritic cells (DC)	Stimulation index
HBsAg-immunized mice	PDC-pulsed DC	1.0
	HBsAg-pulsed DC	15 ± 3.2*
	HBcAg-pulsed DC	1.7 ± 0.5
HBcAg-immunized mice	PDC-pulsed DC	1.0
	HBsAg-pulsed DC	1.9 ± 0.5
	HBcAg-pulsed DC	31 ± 5.2*
PDC-immunized mice	HBsAg-pulsed DC	1.0
	HBcAg-pulsed DC	1.8 ± 0.6
	PDC-pulsed DC	9.2 ± 2*

Normal C57BL/6J mice were immunized with HBsAg, HBcAg, and PDC. Antigen-pulsed DC were prepared by culturing DC with different antigens, as described in the Section 2. Mice were sacrificed 4 weeks after the second immunization, and spleen cells were stimulated with different types of DC. The levels of blastogenesis in cultures containing T cells and irrelevant antigen-pulsed DC were regarded to as a stimulation index of 1.0. Data are presented as mean and standard deviation of five separate experiments. Stimulation index > 3.0 was regarded to as significant antigen-specific proliferation. *<0.05 vs.T cells stimulated with irrelevant antigen-pulsed DC.

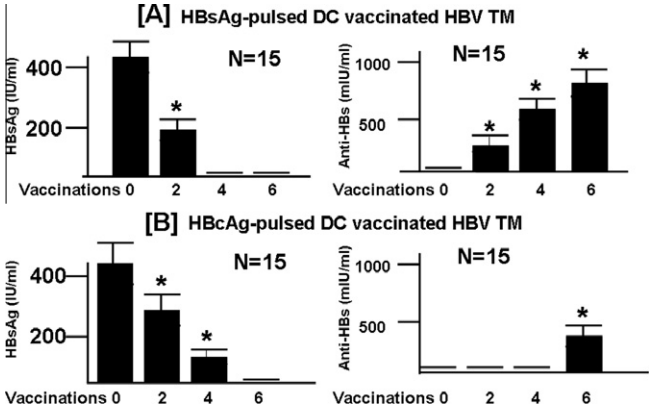

Fig. 1. HBsAg negativity and development of anti-HBs in HBV TM immunized with (a) HBsAg-pulsed DC ($n = 15$) and (B) HBcAg-pulsed DC ($n = 15$). HBsAg and anti-HBs were estimated in the sera by chemiluminescence enzyme immunoassay and expressed as IU/ml and mIU/ml, respectively. Data are presented as mean and standard deviation. 0, 2, 4, and 6 represent before vaccination, after two vaccinations, after four vaccinations and after six vaccinations, respectively. $^{*}p < 0.05$ compared to before vaccination (0).

Additionally, anti-HBs were not detected in HBV TM immunized with unpulsed DC and PDC-pulsed DC (data not shown).

HBsAg and anti-HBs levels at different time points after administration of antigen-pulsed DC are presented in Fig. 1. Eight of the 15 HBV TM immunized with HBsAg-pulsed DC became negative for HBsAg after two vaccinations, and all HBV TM became negative for HBsAg after four vaccinations. High levels of anti-HBs were induced in HBV TM after six vaccinations with HBAg-pulsed DC (Fig. 1A).

Interestingly, immunization with HBcAg-pulsed DC also resulted in a downregulation of HBsAg in HBV TM. All HBV TM immunized with HBcAg-pulsed DC became negative for HBsAg after six vaccinations. Anti-HBs were also detected in all HBV TM immunized with six vaccinations with HBcAg-pulsed DC (Fig. 1B).

3.3. Proliferation of antigen-specific spleen T lymphocytes in HBV TM due to immunization with antigen-pulsed DC

In order to develop insights about role of antigen-pulsed DC on proliferative responses of T lymphocytes, *in vitro* studies were accomplished with spleen T lymphocytes of HBV TM. Six vaccinations with unpulsed DC or PDC-pulsed DC did not induce HBsAg- and HBcAg-specific lymphocytes in HBV TM, as the spleen T lymphocytes of these mice did not show significant proliferation following stimulation with HBsAg or HBcAg *in vitro* (data not shown).

Antigen-specific proliferation of spleen T lymphocytes in HBV TM immunized with HBsAg-pulsed DC is presented in Table 2, panel A. The proliferation levels of T lymphocytes before vaccination were considered a stimulation index of 1.0. T lymphocytes from the spleen of HBV TM immunized with HBsAg-pulsed DC exhibited significant T cell proliferative responses to HBsAg (stimulation index 17.4 ± 4.3, $n = 5$). However, T lymphocytes of HBV TM immunized with HBsAg-pulsed DC did not demonstrate any HBcAg-specific immune responses (Table 1, panel A). Conversely, spleen T lymphocytes from HBV TM immunized with HBcAg-pulsed DC showed significant levels of proliferation in response to both HBsAg (stimulation index, 28.4 ± 3.8, $N = 5$) and HBcAg (stimulation index, 41.2 ± 4.1, $N = 5$).

3.4. Immunization of HBV TM with HBcAg-pulsed DC induced HBcAg- and HBsAg-specific IFN-γ producing $CD8^+$ cytotoxic T lymphocytes (CTL) in the liver

To compare the capacities of HBsAg-pulsed DC or HBcAg-pulsed DC to induce antigen-specific immune responses in the liver of HBV TM, HBsAg-specific and HBcAg-specific CTL were enumerated among liver NPC. Immunization of HBV TM with unpulsed DC or PDC-pulsed DC did not induce significant numbers of CTL (i.e., IFN-γ producing $CD8^+$ T) in the liver following stimulation with HBsAg or HBcAg. Considerable numbers of CTL were detected among liver $CD8^+$ T lymphocytes of HBV TM immunized with HBsAg-pulsed DC following stimulation with HBsAg *in vitro* (213 ± 23, $N = 5$) but not with HBcAg (17 ± 6, $N = 5$) (Table 2, panel B). Conversely, very high proportions of both HBcAg-specific $CD8^+$ CTL (623 ± 38, $N = 5$) and HBsAg-specific $CD8^+$ CTL (453 ± 32, $N = 5$) were detected in the liver of HBV TM immunized with HBcAg-pulsed DC (Table 2, panel B).

Table 2
Antigen-specific T cells in the spleen and the liver of HBV TM immunized with antigen-pulsed DC.

HBV TM immunized with	HBsAg-specific T cell proliferation	HBcAg-specific T cells proliferation
(A) *Antigen-specific T cells in the spleen of HBV TM immunized with antigen-pulsed DC*		
HBsAg-pulsed DC	17.4 ± 4.3	1.2 ± 0.4
HBcAg-pulsed DC	28.4 ± 3.8	41.2 ± 4.1
HBV TM immunized with	HBsAg-specific ELISPOT	HBcAg-specific ELISPOT
(B) *IFN-γ-secreting CD8 + T-cells in the liver of HBV TM immunized with antigen-pulsed DC*		
Unpulsed DC	13 ± 4	11 ± 3
PDC pulsed DC	13 ± 3	12 ± 3
HBsAg-pulsed DC	213 ± 23*	17 ± 6
HBcAg-pulsed DC	453 ± 32*	623 ± 38*

Hepatitis B virus (HBV) transgenic mice (TM) were injected with unpulsed DC, pyruvate dehydrogenase complex (PDC)-pulsed DC, hepatitis B surface antigen (HBsAg)-pulsed DC, or hepatitis B core antigen (HBcAg)-pulsed DC, six times every 2 weeks. HBV TM were sacrificed 2 weeks after the last vaccination.
Panel A: Spleen T cells were evaluated for antigen-specific proliferation in lymphoproliferative assay. The proliferation levels of T lymphocytes before vaccination were regarded as stimulation index of 1.0. Data are presented as mean and standard deviation of five separate experiments.
Panel B: Liver $CD8^+$ T cells were stimulated with HBsAg or HBcAg on an ELISPOT plate to assay IFN-γ production. Data are presented as mean and standard deviation of five separate experiments. *<0.05 vs. HBV TM immunized with unpulsed DC or PDC-pulsed DC.

3.5. Increased production of proinflammatory cytokines by liver NPC from HBV TM immunized with HBcAg-pulsed DC compared to those immunized with HBsAg-pulsed DC

To evaluate cytokine production by liver NPC from HBV TM immunized with HBsAg-pulsed DC or HBcAg-pulsed DC, Liver NPC from HBV TM immunized with HBsAg- or HBcAg-pulsed DC were cultured with HBsAg or HBcAg. IFN-γ and TNF-α levels were significantly higher in HBV TM immunized with HBsAg- or HBcAg-pulsed DC compared to unpulsed HBV TM ($p < 0.05$) (Fig. 2). However, the levels of both cytokines were significantly higher in HBV TM immunized with HBcAg-pulsed DC compared to those immunized with HBsAg-pulsed DC ($p < 0.05$) (Fig. 2).

3.6. Increased activation of endogenous DC via administration of HBcAg-pulsed DC in HBV TM

Although antigen-pulsed DC induced antigen-specific T lymphocytes in HBV TM, it was necessary to assess if antigen-pulsed DC can activate endogenous DC of these mice. Four weeks after six vaccinations with antigen-pulsed DC in HBV TM, DC were isolated from HBV TM to assess the functional capacities of endogenous DC. DC were cultured with allogenic T lymphocytes from

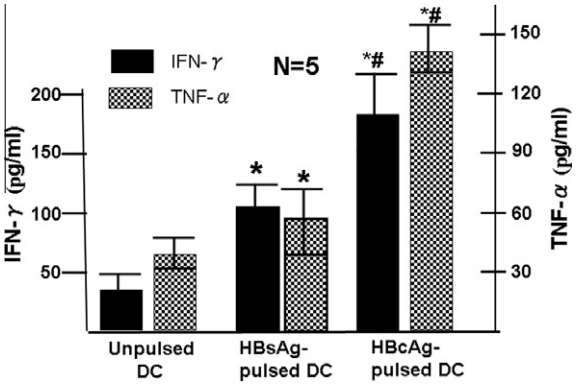

Fig. 2. Significantly higher levels of IFN-γ and TNF-α production via liver NPC in HBV TM immunized with HBcAg-pulsed DC compared to those immunized with HBsAg-pulsed DC. Liver NPC from HBV TM immunized with unpulsed DC or HBsAg-pulsed DC or HBcAg-pulsed DC were cultured with HBsAg or HBcAg and the levels of IFN-γ and TNF-α were estimated by CBA method. Mean and standard deviation of IFN-γ (black bar) and TNF-α (checkered bar) levels produced via liver NPC following immunization with unpulsed DC, HBsAg-pulsed DC, and HBcAg-pulsed DC in five separate experiments. $^{*}p < 0.05$ vs. HBV TM immunized with unpulsed DC. $^{\#}p < 0.05$ vs. HBV TM immunized with HBsAg-pulsed DC.

C3H/He mice, and T cell proliferation levels in allogenic MLR and cytokines in culture supernatants were estimated. The allostimulatory capacities of DC were significantly higher in HBV TM immunized with HBsAg- or HBcAg-pulsed DC compared to DC from HBV TM immunized with unpulsed DC (Fig. 3). HBV TM immunized with HBcAg-pulsed DC showed significantly higher T cell proliferation levels than DC from HBV TM immunized with HBsAg-pulsed DC (Fig. 3A).

As shown in Fig. 3B, IFN-γ and TNF-α levels were also significantly higher in culture supernatants of allogenic MLR containing DC from HBV TM immunized with HBsAg- or HBcAg-pulsed DC compared to those containing DC from HBV TM immunized with unpulsed DC ($p < 0.05$). The levels of IFN-γ were significantly higher in cultures containing DC from HBcAg-pulsed immunized HBV TM compared to HBsAg-pulsed immunized HBV TM ($p < 0.05$).

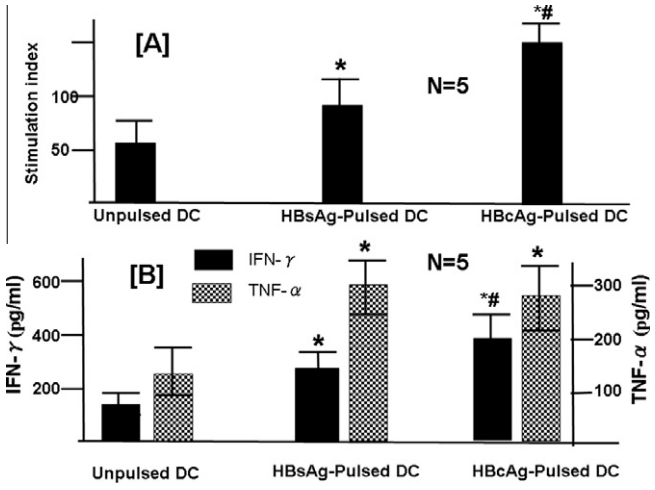

Fig. 3. Increased immunogenicity of DC in HBV TM immunized with HBcAg-pulsed DC compared to HBV TM immunized with HBsAg-pulsed DC. DC were cultured with allogenic T lymphocytes from C3H/He mice, and T cell proliferation levels in allogenic MLR and cytokines in culture supernatants were estimated. (A) Allostimulatory capacity of DC from HBV TM immunized with unpulsed DC or HBsAg-pulsed DC or HBcAg-pulsed DC. (B) Production of IFN-γ (Black bar) and TNF-α (checkered bar) in culture containing DC from HBV TM immunized with unpulsed DC or HBsAg-pulsed DC, or HBcAg-pulsed DC. Data are presented as mean and standard deviation of the stimulation index and cytokines in five separate experiments. $^{*}p < 0.05$ vs. HBV TM immunized with unpulsed DC. $^{\#}p < 0.05$ vs. HBV TM immunized with HBsAg-pulsed DC.

4. Discussion

Antigen-based immune therapy (vaccine therapy) has emerged as a potential therapeutic approach for CHB patients, as it is based on the concept of controlling HBV replication and preventing liver damage in CHB by inducing and maintaining HBV-specific immune responses. Investigators have shown that non HBV-specific immune responses are mainly responsible for impaired control of HBV replication and progressive liver damages in CHB patients, whereas, HBV-specific immune responses, especially HBcAg-specific CTL, are related to control of HBV replication and containment of liver damages in CHB patients (Bertoletti and Maini, 2000). CHB patients that are capable of controlling HBV replication and liver damage harbor higher frequencies of HBV-specific immunocytes, especially HBcAg-specific CTL, compared to those that express high levels of HBV and have progressive liver damage (Maini et al., 2000). These facts show that vaccine therapy can be regarded as an evidence-based therapeutic approach for CHB patients, however, there is still controversy regarding the therapeutic strategies of using vaccines. Although several variables may be important in this context, such as the nature of the antigen, dose of antigen, duration of therapy, and nature of adjuvant, it is of utmost importance to develop further insight regarding the nature of antigens that are proposed to be used as therapeutic vaccines in CHB. After the first clinical trial on HBsAg-based vaccine therapy conducted by Pol et al. (1994), several studies during the last 15 years have pointed to the inherent limitations of HBsAg-based vaccines, even if these therapies are given in combination with antiviral drugs (Pol et al., 2001; Vandepapelière et al., 2007) or loaded on DC or other immunocytes (Akbar et al., 2010a). Both Hoa et al. (2009) and Akbar et al. (2010a) found that HBsAg-based vaccine therapy induced HBsAg-specific immunity and anti-HBs in some CHB patients, but these were not translated into therapeutic efficacy, as adequate levels of HBcAg-specific immunity was not induced.

The data from these studies suggests that HBcAg-pulsed DC are capable of: (1) inducing HBsAg negativity in the sera, (2) developing anti-HBs in the sera, and (3) inducing both HBsAg and HBcAg-specific T cells and CTL in the spleen and the liver. The induction of HBcAg-specific immunity was expected in HBV TM immunized with HBcAg-pulsed DC. However, the effects of HBcAg-pulsed DC on HBsAg-specific immunity in HBV TM is worthy of consideration in the context of immune therapy. Our data corroborates previous reports on the wide-spread immunomodulatory capacities of HBcAg as an adjuvant to HBsAg-specific immunity (Lobaina et al., 2005; Aguilar et al., 2004).

In the present study, we also explored the mechanisms underlying the immunomodulatory effects of HBcAg. It was found that the strong immunomodulatory capabilities of HBcAg may be due to an establishment of an inflammatory hepatic microenvironment, induction of HBcAg-specific CTL in the liver, and activation of host DC. Lee et al. (2009) has reported that HBcAg activates innate immunity. Although we have not checked activation levels of cells of innate immunity in this study, increased production of pro-inflammatory cytokines by liver NPC of HBV TM immunized with HBcAg-pulsed DC compared to those immunized with HBsAg-pulsed DC provide an indirect support for the notion that innate immunity may be stimulated by HBcAg. However, this remains to be confirmed in future in more details.

Taken together, HBcAg should be an integral part of a therapeutic vaccine against chronic HBV infection. However, factors, such as the dose of antigen and duration of therapy, should be properly determined prior to the development of therapeutic vaccines

against CHB. In a previous report (Akbar et al., 2010b), we could not induce HBsAg-specific immune responses by HBcAg-based immunization by loading spleen DC with 10 μg of HBcAg and administrating 2 million DC twice. In this study, we loaded DC with 50 μg of HBcAg and administered 5 million HBcAg-pulsed DC six times. Thus, additional studies would be required to determine the influence of factors, such as dose and duration of therapy, in restoring immunity in CHB.

The clinical utility of the data presented herein may not be translatable to the human condition, as there are fundamental differences between HBV TM and CHB patients. In addition, HBV TM do not demonstrate all of the different features of HBV-related pathogenesis, as they have no evidence of liver injury and exhibit very low levels or almost no circulating HBV DNA. Thus, the implications of these findings need to be confirmed in CHB patients, and the role of HBcAg should be further assessed in humans. The major limitation of the present study lies in the fact that human consumable and commercially developed HBcAg are seldom available for clinical trials in human. To address this issue, we have been conducting a clinical trial with a human consumable HBcAg/HBsAg conjugate vaccine in CHB patients. Preliminary outcomes suggest that the HBsAg/HBcAg-based vaccine induces HBV negativity in 50% of subjects, diminishes liver damage in almost all of the patients, and induces HBsAg- and HBcAg-specific immune responses (Akbar et al., 2010c). However, the relative contribution of HBsAg and HBcAg in this protocol could not be assessed properly. A future study has been designed in which only human consumable HBcAg will be used as a therapeutic vaccine in CHB.

It is still unclear whether HBcAg-based or a conjugate vaccine containing both HBcAg and HBsAg may be required in the design of an evidence-based immunotherapeutic approach against CHB. The findings of the present study, as well as the clinical observations with the HBcAg/HBsAg-based vaccine (Akbar et al., 2010c) in CHB, indicate that HBcAg should be an integral part of a therapeutic vaccine against CHB.

References

Aguilar, J.C., Lobaina, Y., Muzio, V., García, D., Pentón, E., Iglesias, E., Pichardo, D., Urquiza, D., Rodríguez, D., Silva, D., Petrovsky, N., Guillén, G., 2004. Development of a nasal vaccine for chronic hepatitis B infection that uses the ability of hepatitis B core antigen to stimulate a strong Th1 response against hepatitis B surface antigen. Immunol. Cell. Biol. 82, 539–546.

Akbar, S.M., Furukawa, S., Horiike, N., Abe, M., Hiasa, Y., Onji, M., 2010a. Safety and immunogenecity of hepatitis B surface antigen-pulsed dendritic cells in patients with chronic hepatitis B. J. Viral. Hepat. 18, 408–414.

Akbar, S.M., Yoshida, O., Chen, S., Aguilar, A.J., Abe, M., Matsuura, B., Hiasa, Y., Onji, M., 2010b. Immune modulator and antiviral potential of dendritic cells pulsed with both hepatitis B surface antigen and core antigen for treating chronic HBV infection. Antivir. Ther. 15, 887–895.

Akbar, S.M., Al-Mahtab, M., Rahman, S., Aguilar, J.C., Onji, M., Mishiro, S., 2010c. Therapeutic potential of a novel therapeutic vaccine containing both hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) administered through mucosal and parental route in patients with chronic hepatitis B. Hepatology 52 (Suppl.), 438A–439A.

Araki, K., Miyazaki, J., Hino, O., Tomita, N., Chisaka, O., Matsubara, K., Yamamura, K., 1989. Expression and replication of hepatitis B virus genome in transgenic mice. Proc. Natl. Acad. Sci. USA 86, 207–211.

Banchereau, J., Palucka, A.K., 2005. Dendritic cells as therapeutic vaccines against cancer. Nat. Rev. Immunol. 5, 296–306.

Bertoletti, A., Maini, M.K., 2000. Protection or damage: a dual role for the virus-specific cytotoxic T lymphocyte response in hepatitis B and C infection? Curr. Opin. Microbiol. 3, 387–392.

Chen, S., Akbar, S.M., Abe, M., Hiasa, Y., Onji, M., 2011. Immunosuppressive functions of hepatic myeloid-derived suppressor cells of normal mice and in a murine model of chronic hepatitis B virus. Clin. Exp. Immunol. 166, 134–142.

Heathcote, J., McHutchison, J., Lee, S., Tong, M., Benner, K., Minuk, G., Wright, T., Fikes, J., Livingston, B., Sette, A., Chestnut, R.The CY1899 T Cell Vaccine Study Group, 1999. A pilot study of the CY-1899 T-cell vaccine in subjects chronically infected with hepatitis B virus. Hepatology 30, 531–536.

Hoa, P.T., Huy, N.T., Thu le, T., Nga, C.N., Nakao, K., Eguchi, K., Chi, N.H., Hoang, B.H., Hirayama, K., 2009. Randomized controlled study investigating viral suppression and serological response following pre-S1/pre-S2/S vaccine therapy combined with lamivudine treatment in HBeAg-positive patients with chronic hepatitis B. Antimicrob. Agents Chemother. 53, 5134–5140.

Lee, B.O., Tucker, A., Frelin, L., Sallberg, M., Jones, J., Peters, C., Hughes, J., Whitacre, D., Darsow, B., Peterson, D.L., Milich, D.R., 2009. Interaction of the hepatitis B core antigen and the innate immune system. J. Immunol. 182, 6670–6681.

Liaw, Y.F., 2009. Natural history of chronic hepatitis B virus infection and long-term outcome under treatment. Liver Int. 29 (Suppl 1), 100–107.

Liaw, Y.F., Chu, C.M., 2009. Hepatitis B virus infection. Lancet 373, 582–592.

Lin, S.M., Sheen, I.S., Chien, R.N., Chu, C.M., Liaw, Y.F., 1999. Long-term beneficial effect of interferon therapy in patients with chronic hepatitis B virus infection. Hepatology 29, 971–975.

Lobaina, Y., Palenzuela, D., Pichardo, D., Muzio, V., Guillén, G., Aguilar, J.C., 2005. Immunological characterization of two hepatitis B core antigen variants and their immunoenhancing effect on co-delivered hepatitis B surface antigen. Mol. Immunol. 42, 289–294.

Lok, A.S., McMahon, B.J., 2007. Chronic hepatitis B. Hepatology 45, 507–539.

Luo, J., Li, J., Chen, R.L., Nie, L., Huang, J., Liu, Z.W., Luo, L., Yan, X.J., 2010. Autologus dendritic cell vaccine for chronic hepatitis B carriers: a pilot, open label, clinical trial in human volunteers. Vaccine 28, 2497–2504.

Maini, M.K., Boni, C., Lee, C.K., Larrubia, J.R., Reignat, S., Ogg, G.S., King, A.S., Herberg, J., Gilson, R., Alisa, A., Williams, R., Vergani, D., Naoumov, N.V., Ferrari, C., Bertoletti, A., 2000. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. J. Exp. Med. 191, 1269–1280.

Miyake, T., Akbar, S.M., Yoshida, O., Chen, S., Hiasa, Y., Matsuura, B., Abe, M., Onji, M., 2010. Impaired dendritic cell functions disrupt antigen-specific adaptive immune responses in mice with non alcoholic fatty liver disease. J. Gastroenterol. 45, 859–867.

Pol, S., Driss, F., Michel, M.L., Nalpas, B., Berthelot, P., Brechot, C., 1994. Specific vaccine therapy in chronic hepatitis B infection. Lancet 344 (8918), 342.

Pol, S., Nalpas, B., Driss, F., Michel, M.L., Tiollais, P., Denis, J., Brécho, C., 2001. Multicenter study group. Efficacy and limitations of a specific immunotherapy in chronic hepatitis B. J. Hepatol. 34, 917–921.

Shamliyan, T.A., MacDonald, R., Shaukat, A., Taylor, B.C., Yuan, J.M., Johnson, J.R., Tacklind, J., Rutks, I., Kane, R.L., Wilt, T.J., 2009. Antiviral therapy for adults with chronic hepatitis B: A systemic review for a National Institute of Health consensus development conference. Ann. Intern. Med. 150, 111–124.

Sprengers, D., Janssen, H.L., 2005. Immunomodulatory therapy for chronic hepatitis B virus infection. Fund Clin. Pharmacol. 19, 17–26.

Steinman, R.M., Banchereau, J., 2007. Taking dendritic cells into medicine. Nature 449, 419–426.

van der Molen, R.G., Sprengers, D., Binda, R.S., de Jong, E.C., Niesters, H.G., Kusters, J.G., Kwekkeboom, J., Janssen, H.L., 2004. Functional impairment of myeloid and plasmacytoid dendritic cells of patients with chronic hepatitis B. Hepatology 40, 738–746.

Vandepapelière, P., Lau, G.K., Leroux-Roels, G., Horsmans, Y., Gane, E., Tawandee, T., Merican, M.I., Win, K.M., Trepo, C., Cooksley, G., Wettendorff, M., Ferrari, C., 2007. Therapeutic HBV Vaccine Group of Investigators. Therapeutic vaccination of chronic hepatitis B patients with virus suppression by antiviral therapy: a randomized, controlled study of co-administration of HBsAg/AS02 candidate vaccine and lamivudine. Vaccine 25, 8585–8597.

Wang, X.Y., Zhang, X.X., Yao, X., Jiang, J.H., Xie, Y.H., Yuan, Z.H., Wen, Y.M., 2010. Serum HBeAg sero-conversion correlated with decrease of HBsAg and HBV DNA in chronic hepatitis B patients treated with a therapeutic vaccine. Vaccine 28, 8169–8174.

Wilt, T.J., Shamliyan, T., Shaukat, A., Taylor, B.C., MacDonald, R., Yuan, J.M., Johnson, J.R., Tacklind, J., Rutks, I., Kane, R.L., 2008. Management of chronic hepatitis B. Evid. Rep. Technol. Assess (Full Rep.) 174, 1–671.

Yoshida, O., Akbar, S.M., Chen, S., Miyake, T., Abe, M., Hiasa, Y., Murakami, H., Onji, M., 2010. Regulatory natural killer cells in murine liver and their immunosuppressive capacity. Liver Int. 30, 906–912.