

## Effects of lamivudine on the function of dendritic cells derived from patients with chronic hepatitis B virus infection

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**CONCLUSION:** The lower expression of phenotypic molecules and impaired allogeneic mixed lymphocyte reaction function of dendritic cells derived from patients with HBV infection could be restored *in vitro* by incubation with LAM.

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**Key words:** Dendritic cell; Lamivudine; Chronic hepatitis B; Immune

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### Abstract

**AIM:** To investigate if the nucleoside analogue lamivudine (LAM), a potent inhibitor of HBV replication, could restore the function of dendritic cells derived from patients with chronic hepatitis B (CHB) in an Asian population.

**METHODS:** Dendritic cells (DCs) derived from mononuclearcytes of patients with chronic HBV infection were cultured in the presence of IL-4, granulocyte-macrophage colony-stimulating factors (GM-CSF) and gradient concentrations of LAM (0-2 mmol/L). Cell morphology was observed under light microscopy. Cell surface molecules, including HLA-DR, CD80, CD83, and CD1 $\alpha$ , were analyzed with flow cytometry. The concentrations of IL-6 and IL-12 in the supernatant were assayed by ELISA. T cell proliferation was assayed by methyl thiazolyl tetrazolium (MTT).

**RESULTS:** The expression of CD1 $\alpha$  on DC treated with 0.5 mmol/L LAM (LAM-DC 0.5 mmol/L) was significantly higher than that of DC untreated with LAM ( $54.1 \pm 4.21$  vs  $33.57 \pm 3.14$ ,  $P < 0.05$ ), and so was the expression of CD83 ( $20.24 \pm 2.51$  vs  $12.83 \pm 2.12$ ,  $P < 0.05$ ) as well as the expression of HLA-DR ( $74.5 \pm 5.16$  vs  $52.8 \pm 2.51$ ,  $P < 0.05$ ). Compared with control group, LAM-DC group (0.5 mmol/L) secreted significantly more IL-12 ( $910 \pm 91.5$  vs  $268 \pm 34.3$  pg/mL,  $P < 0.05$ ), had lower levels of IL-6 in the culture supernatant ( $28 \pm 2.6$  vs  $55 \pm 7.36$  pg/mL,  $P < 0.05$ ), markedly enhanced the stimulatory capacity in the allogeneic mixed leukocyte reaction (MLR) ( $1.87 \pm 0.6$  vs  $1.24 \pm 0.51$ ,  $P < 0.05$ ).

### INTRODUCTION

More than 300 million people worldwide are chronically infected with hepatitis B virus (HBV), and the majority of them are in Asia, especially in China. One of the important mechanisms resulting in the immune tolerance by chronic hepatitis B (CHB) is the impaired function of dendritic cells (DCs) derived from patients with CHB<sup>[1]</sup>. DCs are antigen-presenting cells (APC) responsible for initiating immunity and play an important role in the induction of antiviral immune responses<sup>[2]</sup>. Recently there has been considerable interest in using DCs as adjuvants to enhance host immunity against viral infection<sup>[3]</sup>.

Lamivudine (LAM), a nucleoside analogue, is a potent and selective inhibitor of HBV replication. LAM specifically inhibits hepadnaviral DNA polymerase by competing with the corresponding dNTPs for incorporation into nascent DNA acting as a chain terminator after incorporation<sup>[4]</sup>. In addition to anti-HBV replication, the potential role of LAM in the regulation of immune response in HBV patients has been noticed recently. LAM treatment can restore T cell responsiveness in chronic hepatitis B<sup>[5]</sup>. Furthermore, it has been reported that LAM could up-regulate major histocompatibility complex class II (MHC-II) expression and restore impaired allostimulatory function of mononuclear cell-derived DC in the Caucasian patients with HBV infection<sup>[6]</sup>. In China, the influence of LAM on function of DC has not been investigated. We, therefore, HBsAg-pulsed DCs derived from the mononuclear cells

of patients with chronic HBV infection with different LAM concentrations (0-2 mmol/L) *in vitro* and observed whether LAM could enhance DCs function. We attempted to explore a new approach to combine LAM and dendritic cell-based immunotherapy for CHB infection.

## MATERIALS AND METHODS

### Materials

rhGM-CSF, rhIL-4, mouse anti-human HLA-DR-PE, CD80-FITC, CD1 $\alpha$ -FITC, CD83-PE were purchased from BioLegend. RPMI-1640 was purchased from GIBCO (USA), and fetal calf serum (FCS) from Hangzhou Sijiqing Biological Engineering, China. Ficoll-Hypaque density gradient separate solution was purchased from Tianjin Jinmai Gene Biotechnology Co., China. rhIL-6, IL-12 ELISA Kit (Peprotech) were purchased from Shanghai Shenxiong Technology Company, China. LAM was purchased from GlaxoSmithKline Company.

### Subjects

Fifteen outpatients with HBeAg-positive CHB infection participated in this study. Written informed consent was obtained from all the patients and the study protocol was approved by the Ethics Committee of Zhengzhou University.

### Preparation of dendritic cells

Peripheral blood mononuclear cells (PBMCs) were prepared from CHB patients. Briefly, PBMCs were washed and resuspended at  $2 \times 10^6$  cells/mL. The cells were cultured in RPMI 1640 media containing 100 mL/L autologous serum, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin. After incubation for 2 h at 37°C, non-adherent cells were removed; the adherent cells were cultured with RPMI 1640 media with recombinant GM-CSF and IL-4 in a 24-well culture plate at 37°C in a humidified atmosphere containing 50 mL/L CO<sub>2</sub>. Mononuclear cells were assigned to two groups on d 3: LAM-treated group (experimental group), and non-LAM-treated group (control group). The experimental group was further divided into subgroups according to the different concentrations (0.125, 0.25, 0.5, 1, 2 mmol/L) of LAM. Half of the medium was replaced with fresh medium every other day. DCs were harvested on the 8<sup>th</sup> d.

### Analysis of DC morphology and flow cytometry (FACS)

DCs were observed on an inverted microscope dynamically, and the phenotypes of DCs, such as CD1a, CD80, CD83 and HLA-DR, were analyzed by FACS on the 8<sup>th</sup> day. FITC-labelled mouse anti-human CD1 $\alpha$ , CD80 monoclonal antibody (mAb) and phycoerythrin-conjugated mouse anti-human CD83, HLA-DR mAb were used. Staining was performed as described previously<sup>[7]</sup>.

### Allogeneic mixed leukocyte reaction

To clarify whether the antigen-presenting capacity of LAM-treated DCs (LAM-DC) was different from that of non-LAM-treated DCs (non-LAM-DC), mononuclear cells were isolated from the peripheral blood of healthy

subjects. After incubation for 2 h, the non-adherent cells were collected as lymphocyte cells. DCs were collected as stimulator cells at a concentration of  $1 \times 10^5$ /mL and added into a 96-well culture plate. DCs were incubated with mitomycin C (50  $\mu$ g/mL) for 30 min, and then lymphocytes were added as responder cells at a concentration of  $1 \times 10^6$ /mL; each group was set to triple wells. After being cultured for 3 d, absorbance at 570 nm ( $A_{570}$ ) was assayed by MTT and the stimulator index (SI) was calculated using a formula:  $SI = A_{\text{experiment}} / (A_{\text{responder cells}} + A_{\text{stimulator cells}})$ .

### Evaluation of IL-12 and IL-6 in supernatant of DC

Levels of IL-12 and IL-6 in the supernatant of DC were determined using an enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's instructions. Samples of each group were set to triple wells.

### Statistical analysis

Data were analyzed with SPSS10.0 statistical software. The significant difference between groups was determined by one-way ANOVA test. Two-sided  $P < 0.05$  was considered statistically significant.

## RESULTS

### Morphology analyses

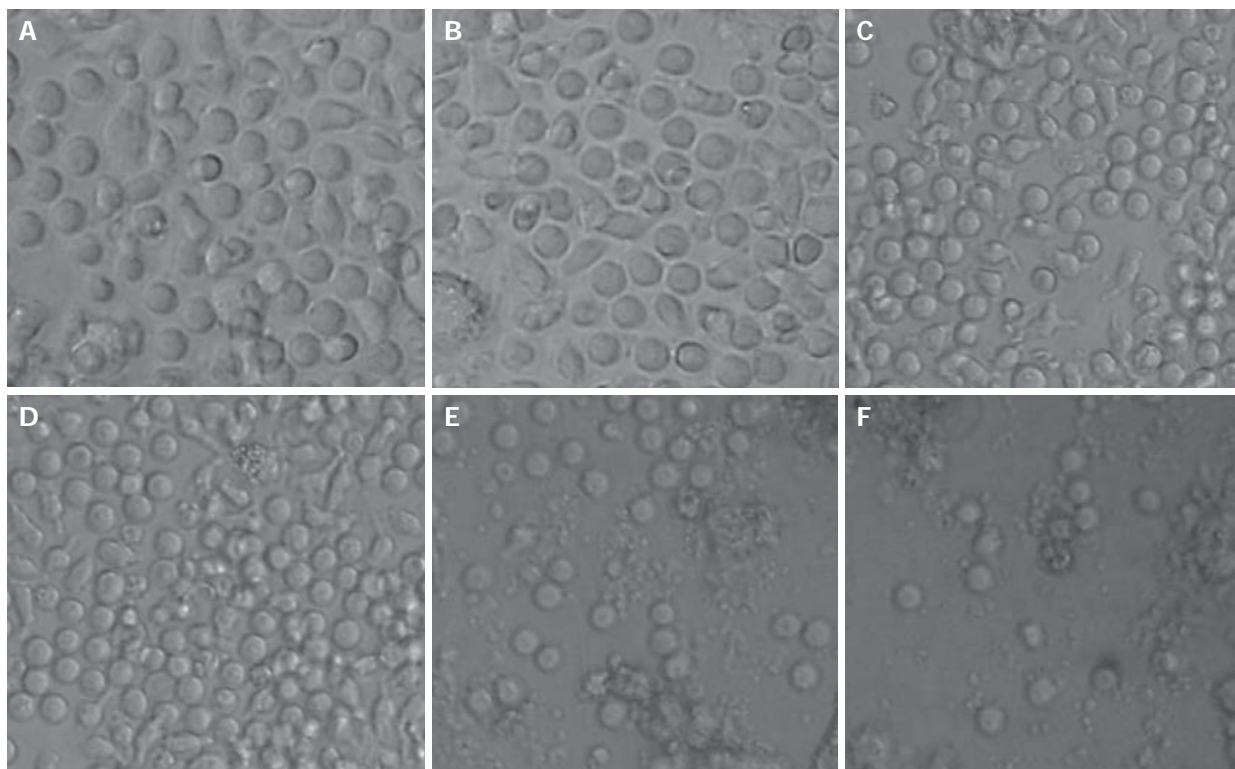
After being cultured for 24 h, DCs began to grow as fully differentiated swarm cells as observed under a microscope; 3 d later there was an increase in size and in numbers of DCs; 6 d later much salience on the surface of DCs was observed; many nebulous substance floating among culture media were also found on the 8<sup>th</sup> day. However, the LAM-DC at low doses had no distinct difference in morphology from control group (Figure 1A and B). There was an increase in number of grainy substance in culture media of the LAM-DC at a high dose on the 2<sup>nd</sup> day (Figure 1F), which might be cell debris resulted from the medication toxicity.

### Phenotype of dendritic cells

The phenotypes of DCs were determined by FACS on the 8<sup>th</sup> day. The expressions of phenotype molecules in DCs diversified among groups treated with different concentrations of LAM. The expressions of CD1 $\alpha$  on LAM-DC (0.5 mmol/L) was significantly higher than those in naïve controls and other groups ( $P < 0.05$ ), and so was the expression of CD83 ( $P < 0.05$ ) and the expression of HLA-DR ( $P < 0.05$ ); there was no difference in CD80 expression between LAM-DC (0.5 mmol/L) group and control group. The results demonstrated that HBsAg-pulsed DCs with LAM at certain concentration can enhance the capacities of antigen-presentation (Table 1).

### Stimulatory capacity of the LAM-DC on lymphocyte proliferation

In the allogeneic mixed leukocyte reaction (MLR), the lymphocyte proliferation reflected the stimulatory function of DCs. The stimulatory capacity of the LAM-DC (0.5 mmol/L) in the allogeneic mixed leukocyte reaction



**Figure 1** Morphology of DCs treated with lamivudine (LAM) at different concentrations on the 8<sup>th</sup> d (× 400). **A:** Non-LAM-treated DC; **B:** DC + LAM (0.125 mmol/L); **C:** DC + LAM (0.25 mmol/L); **D:** DC + LAM (0.5 mmol/L); **E:** DC + LAM (1 mmol/L); **F:** DC + LAM (2 mmol/L).

**Table 1** The expression of DCs disposed by LAM (mean ± SD, n = 15)

	CD83	CD80	CD1α	HLA-DR
DC	12.83 ± 2.12	35.82 ± 2.41	33.57 ± 3.14	52.80 ± 2.51
DC + LAM 0.125 mmol/L	13.40 ± 2.16	37.73 ± 3.24	40.26 ± 2.09	53.70 ± 3.27
DC + LAM 0.25 mmol/L	18.30 ± 3.15	41.13 ± 3.61	53.10 ± 2.79	67.83 ± 4.15
DC + LAM 0.5 mmol/L	20.24 ± 2.51 <sup>a</sup>	41.73 ± 4.18	54.10 ± 4.21 <sup>a</sup>	74.50 ± 5.16 <sup>a</sup>
DC + LAM 1 mmol/L	12.81 ± 2.31	25.66 ± 3.05	16.55 ± 1.95	42.11 ± 3.91
DC + LAM 2 mmol/L	11.92 ± 1.28	24.67 ± 2.62	16.54 ± 1.76	41.01 ± 3.52

DC: Dendritic cell; LAM: Lamivudine. <sup>a</sup>P < 0.05 vs DC + LAM 0 mmol/L group.

(MLR) was markedly enhanced as compared with control group (P < 0.05). On the contrary, there was no significant difference between LAM-DC (0.125 mmol/L) and control group (Table 2).

**Secretion of IL-12 and IL-6 by DCs**

The concentrations of IL-12 in the supernatant of LAM-DC (0, 0.125, 0.25, 0.5, 1, 2 mmol/L) were 268 ± 34.3, 380 ± 51.2, 500 ± 89.4, 910 ± 91.5, 255 ± 73.1, 138 ± 27.9 pg/mL, respectively. The concentrations of IL-6 in the supernatant of LAM-DC (0, 0.125, 0.25, 0.5, 1, 2 mmol/L) were 55 ± 7.36, 47 ± 5.2, 32 ± 2.7, 28 ± 2.6, 69 ± 8.7, 67 ± 8.4 pg/mL, respectively. The secretion of IL-12 significantly increased in the LAM-DC (0.5 mmol/L)

**Table 2** The stimulatory ability of DCs treated with LAM in allogeneic mixed leukocyte reactions (mean ± SD, n = 15)

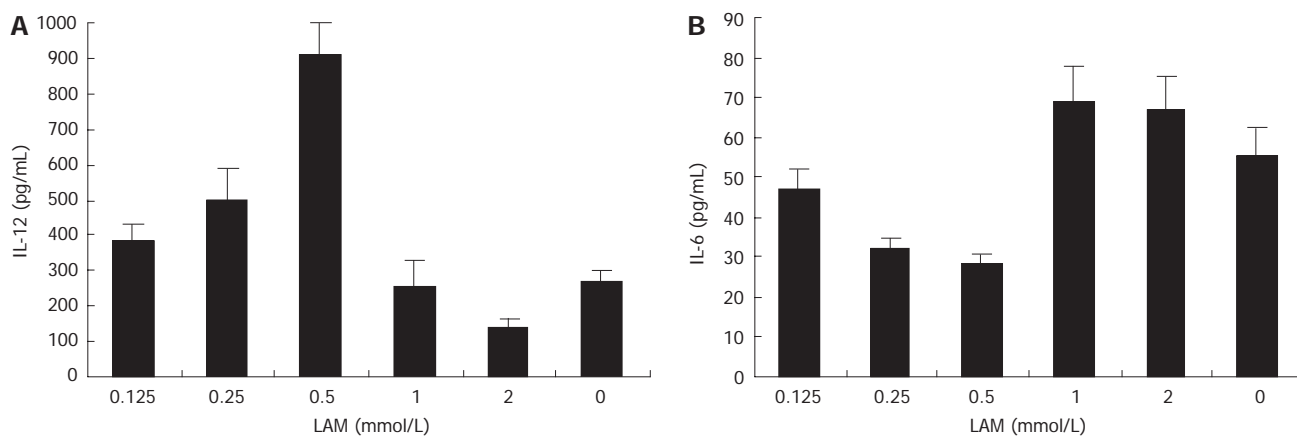
	Control group	LAM (mmol/L)				
		0.125	0.25	0.5	1	2
SI	1.24 ± 0.51	1.31 ± 0.56	1.54 ± 0.45	1.87 ± 0.6 <sup>a</sup>	0.9 ± 0.39	0.63 ± 0.3 <sup>a</sup>

SI: Stimulator index; DC: Dendritic cell; LAM: Lamivudine. <sup>a</sup>P < 0.05 vs control group.

group in comparison with control group (P < 0.05). However, LAM-DC (0.5 mmol/L) group produced a lower level of IL-6 as compared with control group (P < 0.05) (Figure 2).

**DISCUSSION**

Experimental data from HBV-transgenic mice demonstrate that the immune system plays a key role in HBV clearance<sup>[8]</sup>. After HBV infection, the body starts a series of non-specific immunology responses, including activation of natural-killing cells and secretion of interferon. However, the complete clearance of HBV relies on the activation of HBV-specific T lymphocytes<sup>[9]</sup>. The body can produce HBV antigen-specific CTL response to kill target cells related to HBV. DCs are professional antigen-presenting cells that link innate and adaptive immunity, and are essentially involved in the initiation of primary immune responses and in the establishment of peripheral tolerance<sup>[10,11]</sup>. The infection of CHB relates to the impairment of immunity by the mechanism resulting from the deficiency of DC function<sup>[12]</sup>. Selective modification of DC function and promotion of HBV-



**Figure 2** Modulation of cytokine secretion from DCs by LAM treatment. Bars indicate the levels of IL-12 (A) and IL-6 (B) in culture media of DCs treated with LAM at different concentrations (mean  $\pm$  SD,  $n = 15$ ).

specific immune responses during CHB treatment have shown therapeutic significance<sup>[13]</sup>.

DCs could be assigned to two groups by their immunocompetence: mature DCs and immature DCs. Mature DCs have already encountered antigens, whereas immature DCs are able to internalize antigens, process them in the extranuclear compartment, and convert them on the cell surface in the compound of MHC-antigen peptides and present antigenic peptides to T cells. It activates the antigen-specific T cells and initiates immune response with the help of costimulatory molecules binding the corresponding T cell receptors<sup>[14]</sup>. In the present study, we detected the expression of CD1 $\alpha$ , CD80, CD83 and HLA-DR on DCs. CD1 $\alpha$  is the specific sign of DC's function; the more CD1 $\alpha$  expresses on DCs, the strong function DCs have. CD83 is a mature sign of DCs; HLA-DR, one of the MHC II molecules, mostly takes part in the process of antigen presentation; CD80, one of the costimulatory molecules, promotes T cell activation *via* combining the corresponding T cell receptors<sup>[15,16]</sup>.

Beckebaum *et al*<sup>[6]</sup> proposed that LAM could facilitate the maturation of DCs isolated from Caucasian patients with HBV infection by increasing the expression of HLA-DR and further promote DCs function. In the present study, the expression of CD1 $\alpha$ , CD83 and HLA-DR on DCs derived from Chinese patients with CHB infection incubated with LAM (0.5 mmol/L) *in vitro* could be enhanced; however, the increase of CD80 was not statistically different as compared with that of control group. Here we found that the lower expression of phenotypic molecules and impaired allogeneic mixed lymphocyte reaction function of DCs from patients with HBV infection could be restored *in vitro* by incubation with LAM. The present study demonstrated that LAM did promote DCs maturation and enhance DCs function.

DCs regulate Th0 cell proliferation towards Th1 cells and Th2 cells through the secretion of IL-6, IL-12 and IFN- $\gamma$ <sup>[17]</sup>. IL-12 secreted by mature DCs regulates Th0 cells proliferation towards Th1 cells, promotes the secretion of IL-2, IFN- $\gamma$  and participates in the cellular immune response. IL-6 from immature DCs drives Th0 cells to proliferate towards Th2 cells, restrains the cellular

immune and induces the immune tolerance<sup>[18]</sup>. In the current experiment, we found that the secretion levels of IL-12, IL-6 by DCs could be affected by LAM *in vitro*. The secretion of IL-12 increased in the LAM-DC. LAM-DC produced lower levels of IL-6 as compared with non-LAM-DC. These results indicate that DCs treated with LAM at certain concentrations can promote T cell proliferation and enhance the cellular immunity.

The present data confirmed that the defect of DC function could be partially restored by LAM *in vitro* as marked by the enhanced expression of CD1 $\alpha$ , CD83 and HLA-DR on DCs and increased secretion of IL-12, reduced secretion of IL-6 in DCs, and enhanced mixed lymphocyte reaction ability. This indicates that, in addition to its potent anti-HBV replication role, LAM is able to modify the biological activities of DCs derived from patients with CHB infection. Therefore, LAM can be a potential candidate as an immunoregulatory therapeutic remedy used in the treatment of patients with CHB infection. Since treatment with LAM can overcome T cell hyporeactivity<sup>[5]</sup> and restore DCs function, combination of LAM with DCs may be an effective therapeutic strategy to obtain eradication of chronic HBV infection.

## COMMENTS

### Background

Hepatitis B virus (HBV) infection is a global public health problem. The immune response of the host plays an important role in the pathogenesis of chronic HBV infection.

### Research frontiers

One of the important reasons responsive for the immune tolerance in chronic hepatitis B (CHB) is impaired function of dendritic cells (DCs) which cannot efficiently present HBV antigens to host immune system.

### Innovations and breakthroughs

DC-based therapeutic vaccine has recently been a potential approach to treat CHB. Lamivudine (LAM), a nucleoside analogue, specifically inhibits the hepadnaviral DNA polymerase.

### Applications

The present study provides a new support for the application of LAM and DC-based immunotherapy in clinical practice of CHB therapy.

**Peer review**

This is a good paper indicating that combining LAM with DCs may be an effective therapeutic approach to obtain eradication of chronic HBV infection.

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