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Nicotine stimulated bone marrow-derived dendritic cells could augment HBV specific CTL priming by activating PI3K-Akt pathway☆

Hao Jie Jin[a], Hai Tao Li[a], Hua Xiu Sui[a], Mao Qiang Xue[a], Yi Nan Wang[a], Jia Xun Wang[a], Feng Guang Gao[a,b,*]

[a] Department of Immunology, Basic Medicine Science, Medical College, Xiamen University, Xiamen 361005, People's Republic of China
[b] The State Key Laboratory for Oncogenes and Related Genes, Cancer Institute of Shanghai Jiao Tong University, Shanghai 200030, People's Republic of China

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ABSTRACT

Our previous studies have revealed that nicotine-treated immature dendritic cells (imDCs) have anti-tumor effects in murine lymphoma models. The present study is to explore HBV-specific CTL priming and its cytolytic activities of nicotine-treated murine DCs, the mechanism of α7 nicotinic acetylcholine receptor (nAChR) up-regulation by nicotine and the efficiency of nicotine with other cytokines. To address these hypotheses, bone marrow-derived imDCs were stimulated by nicotine and expression of α7 nAChR was firstly determined by flow cytometry and Western blot. Then, DCs-dependent HBV-specific T cell proliferation and IL-12 secretion were secondly determined by BrdU cell proliferation assay and ELISA, respectively. The HBV-specific CTL priming and its activities were further explored by intraperitoneal transfer of nicotine treated imDCs. The mechanism of nicotine up-regulating α7 nAChR was finally explored by Western blot. The results showed that: first, the maximal activation of PI3K and Akt was reached at 30 and 60–120 min respectively after nicotine stimulation. Nicotine up-regulated the expression of α7 nAChR by activating PI3K-Akt pathway in murine DCs; secondly, nicotine stimulation could enhance DCs' ability of HBV-specific T cell proliferation and IL-12 secretion; thirdly, adoptive transfer of nicotine stimulated DCs could induce HBV specific CTL priming *in vivo* and those CTL had cytolytic activities; fourthly, nicotine had equal efficiencies to 2 ng/ml IFN-γ in DCs-mediated T cell proliferation. All these data presented here indicated that nicotine treated imDCs might be considered as a potential candidate for HBV immunotherapy.

1. Introduction

It is estimated that more than 350 million individuals are currently developing lifelong HBV persistence and have a significant risk of progressive liver cirrhosis and primary hepatocellular carcinoma [1]. Dendritic cells (DCs) are the most potent APC with the unique ability to stimulate naive T cells which are key players in the anti-viral immune response [2]. An adaptive immune response is essential for the clearance of viral infection [3–5], but patients with persistent HBV infection have not only impaired T-cell and B-cell responses [6,7], but also impaired DCs' function [8–10]. HBV infection could down-regulate the expression of surface co-stimulatory molecules in DCs and reduce its capacity as APC, which generally fail to trigger efficient cellular immune responses [11,12]. Much efforts have been done to develop therapy for HBV infection, unfortunately, antivirus drugs can pose side effects and have limited efficiency, however there is still no specific curative therapy for HBV infection. Therefore, new immunotherapies should be developed to deal with HBV infection and control the disease progression.

Nicotine, a major component of cigarette smoke, has positive effect in treatment of neurodegenerative diseases and ulcerative colitis [13–15]. The expression of nicotinic acetylcholine receptor (nAChR) has already been demonstrated in many types of non-neuronal cells such as DCs, epithelial cells and endothelial cells [16]. However, the effect of nicotine on immune cells, such as DCs and macrophages, is incompletely characterized and uncertainties still exist. Some investigators have proved that nicotine promotes inflammation [17,18]. Other researchers, by contrast, have provided evidence that nicotine may have immunosuppressive effects,

Abbreviations: imDCs, immature dendritic cells; DCs, dendritic cells; HBV, hepatitis B virus; nAChR, nicotinic acetylcholine receptor; LLC, Lewis lung cancer; Ni, nicotine; BTX, α-bungarotoxin; TC, tubocurarine chloride.

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* Corresponding author at: Department of Immunology, Basic Medicine Science, Medical College, Xiamen University, Xiamen 361005, People's Republic of China. Tel.: +86 592 2186335; fax: +86 592 2182736.
E-mail address: gfengguang@xmu.edu.cn (F.G. Gao).

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although these results were achieved with relatively high doses of nicotine [19,20]. Moreover, nicotine upregulates costimulatory molecules such as CD86 and CD40, MHC class molecules such as HLA-DR, and adhesion molecules such as CD54 and enhances adaptive immunity, an effect that is mediated by nicotinic ACh receptors on human DCs and monocytes [21]. Our previous studies demonstrated that nicotine had stimulatory effects on murine immature DCs (imDCs), enhanced DCs-mediated CTL priming and facilitated the preventive and therapeutic effects on lymphoma [22], Lewis lung cancer and hepatocellular carcinoma [23]. As HBV antigen pulsed DCs can efficiently augment HBV-specific CTL response in chronically infected HBV patients and HBV transgenic mice [24–26], the roles of nicotine-treated DCs in HBV immunotherapy still need to be determined. IFN-γ, IL-15 and IL-18 have recently been reported to augment DCs' functions [27–29]. For this reason, the stimulation efficiencies of nicotine on DCs should be compared with other cytokines such as IFN-γ, when potential clinical implication was considered. Although activations of Jak2 and NF-κB play central roles in α7 nAChR mediated anti-apoptosis and anti-inflammation effects [30], the mechanism of α7 nAChR up-regulation in DCs by nicotine is still unknown and needs further exploration.

In present study, we firstly characterized that nicotine treatment could up-regulate α7 nAChR in imDCs and secondly demonstrated that nicotine stimulation could enhance DCs' ability of HBV-specific T cell proliferation and IL-12 secretion. Thirdly, we revealed that adoptive transfer of nicotine stimulated DCs could induce HBV specific CTL priming *in vivo* and those CTL had cytolytic activities. Fourthly, nicotine treatment had equal efficiencies to 2 ng/ml IFN-γ in DCs-mediated T cell proliferation and IL-12 secretion. Finally, nicotine could up-regulate α7 nAChR by activating PI3K-Akt pathways in murine DCs. All these data presented here indicated that nicotine treated DCs might be considered as a potential candidate for HBV immunotherapy.

2. Materials and methods

2.1. Reagents

Nicotine, α-bungarotoxin, tubocurarine chloride were obtained from Sigma–Aldrich (Missouri, USA). Mouse GM-CSF, IL-4, and IFN-γ were from R&D (Minneapolis, USA). BrdU Cell Proliferation Kit was from Roche (Roche Diagnostics GmbH, Germany). Fluorescene conjugated antibodies were from eBioscience (San Diego, USA). Mouse IL-12 ELISA Kit, IFN-γ ELISPOT Kit, CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit were obtained from Bender MedSystems (Vienna, Austria), U-CyTech Biosciences (Utecht, Netherlands), Promega (Madison, USA), respectively. The p38 MAPK inhibitor SB203580, Jak-2 inhibitor AG490 and PI3K inhibitor LY294002 were from Cayman Chemical (Ann Arbor, USA). The antibodies for Western blot were from Millipore Corporation.

2.2. Animals

Pathogen-free C57BL/6 mice (female, 6–8 weeks old) were bought from Shanghai Laboratory Animal Center of Chinese Academy of Sciences (China) and kept at the Animal Center of Xiamen University. All animal studies were approved by the Review Board, Medical College, Xiamen University.

2.3. Antigens and peptides

Recombinant Hepatitis B surface antigen, Adr subtype (HBsAg), recombinant Hepatitis B virus protein X (HBx), recombinant Hepatitis B virus core (HBV core 1–186), recombinant Hepatitis B virus HBe (HBeAg) were bought from ProSpec-Tany TechnoGene Ltd. The H-2K^b CTL peptides of $HBs_{(335–343)}$ (WLSLLVPFV) [26], $HBe_{(93–100)}$ (MGLKFRQL) [31], $HBx_{(52–60)}$ (HLSLRGLFV) [25] were synthesized by Sangong (Shanghai, China). The purity and toxicity of the peptides were checked by HPLC and electro spray mass spectrometry. Pure peptide fractions were lyophilized and dissolved at 1 mg/ml in PBS.

2.4. Cell line

LLC cell line, established from the lung of C57BL mouse bearing tumor resulting from implantation of primary Lewis lung carcinoma, was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (ATCC CRL-1642). LLC cells were cultured in DMEM with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10% FBS.

2.5. Preparation of murine bone marrow-derived DCs

Bone marrow-derived DCs were prepared as previously described [22]. Briefly, bone marrow cells were cultured in RPMI medium supplemented with 10% FBS at the presence of 10 ng/ml GM-CSF and 1 ng/ml IL-4. After 4 days culture, supernatants were dumped and loosely adherent clusters were washed with PBS and used as imDCs. These DCs were stimulated with 10^{-7} mol/L nicotine for 12 h, or pretreated with 2 μg/ml α-bungarotoxin or 10^{-5} mol/L tubocurarine chloride prior to nicotine stimulation to investigate whether the effects of nicotine were mediated by α7 nAChR. As to compare nicotine's effects with different doses of IFN-γ, DCs were stimulated with recombinant mouse IFN-γ for 12 h at the final concentration of 0.2 ng/ml, 0.5 ng/ml, 1 ng/ml, 2 ng/ml and 5 ng/ml, respectively.

2.6. Flow cytometric measurement

Expression of α7 nAChR was determined by flow cytometry according to the methods described previously [22]. Flow cytometry was done with FACSCalibur and data were analyzed with CellQuest software.

2.7. Antigen-specific T cells proliferation assay

Antigen-specific proliferation assay was performed as described previously [23]. Briefly, imDCs (cultured for 4 days) were treated with 10^{-7} mol/L nicotine for 12 h which further pulsed with HBcAg, $HBe_{(93–100)}$, $HBs_{(335–343)}$ or $HBx_{(52–60)}$ peptide at the concentration of 5 μg/ml respectively for 4 h. Then, cells were collected and washed twice with PBS and used as stimulators. DCs treated with PBS, α-bungarotoxin (2 μg/ml) or TC (10^{-5} mol/L) 60 min prior to nicotine stimulation were used as controls. Responder cells were prepared by depletion of red blood cells from splenocytes of same background C57BL mice. Stimulators were mixed with responders at a ratio of 1:10 in 200 μl volume. After 5 days co-culture, T cell proliferation was determined by BrdU Cell Proliferation assay and the supernatants of MLR was collected for ELISA.

2.8. Quantification of IL-12 production

IL-12 production of MLR were determined by ELISA according to the standard procedure [21]. Briefly, plates were treated with coating antibody at 4 °C overnight, washed with PBS, and blocked with assay buffer at room temperature for 2 h. The blocked plates were washed twice with thorough aspiration of microwell contents between washes. After the last wash step, empty wells and tap microwell strips to remove excess wash buffer. Samples, assay buffer, biotin-conjugate detector antibodies were added to