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Astragalus polysaccharides enhance immune responses of HBV DNA vaccination via promoting the dendritic cell maturation and suppressing Treg frequency in mice

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ABSTRACT

Astragalus polysaccharides (APS), an extract from a kind of Chinese traditional herb *Astragalus membranaceus*, was proved to have strong immunoregulatory properties. In this study, APS was employed as an adjuvant of Hepatitis B virus (HBV) DNA vaccine (pcDS2) and its' effects on immune system of mice were investigated. Our data demonstrated that APS as an adjuvant could increase the HBsAg-specific antibody level as well as the proliferating activity of T cells. APS also could induce $CD4^+$ T cells to produce IL-4, IL-2 and IFN-γ and enhance IFN-γ expression of $CD8^+$ T cells. Moreover, APS could induce the robust activity of the cytotoxic lymphocytes (CTL). Additionally, APS could stimulate the dendritic cells (DC) maturation which is characterized by up-regulation of MHC I/II, CD40, CD80 and CD86, and decreased the frequency of the regulatory T cells (nTreg). Collectively, these findings suggest that APS is a potent adjuvant for the hepatitis B DNA vaccine and can enhance the immune responses of HBV DNA vaccine via promoting DC maturation and inhibit the Treg frequency.

1. Introduction

Hepatitis B is a severely human-threatened disease which will lead to liver cirrhosis and hepatocellular carcinoma (HCC) [1]. The commercial HBV subunit vaccine inoculation is an efficient method to prevent HBV infection, however, exerts little effect on curing the chronic HBV infection. One of important reasons is that this vaccine mainly induces the humoral immune response but very weak cell-mediated immune response which plays the key role in eliminating HBVs [2]. Thus, therapeutic vaccines, which can trigger the strong cellular response, are paid much attention by vaccine researchers. Amongst them, DNA vaccine is one of the most promising candidates.

Though many studies illustrated that DNA vaccine could induce the ideal cellular and humoral immune response [3], certain shortcomings cannot be ignored. DNA vaccine primed insufficient immune response in large animals and humans [4]. Adjuvant can enhance the antigen-specific response as well as extend the effect time. Thus, the adjuvant is regarded as an ideal way to overcome the drawbacks of DNA vaccine, and the search for new adjuvant able to enhance protective immune response is one of the most promising approaches [5].

Astragalus membranaceus, a kind of common Chinese medical herb, was traditionally used to enhance the immunity of human as well as resist certain virus with little side-effect. Its' extract, *Astragalus* polysaccharides (APS), was proved to possess excellent immunopotentiatory property [6]. It has been reported that APS could induce $CD4^+$ T cells to secrete IL-2 and IFN-γ [7]. Also, it could stimulate T and B cell proliferation [8] and activate macrophages [9]. Moreover, APS could prompt DC maturation and its ability of antigen presentation [10]. In our previous study, we have demonstrated that APS can enhance the immunity of recombinant hepatitis B surface antigen, including antibody production, T-cell proliferation and CTL (cytotoxic T lymphocyte) activity [11]. However, the production of recombinant protein antigen is complex and expensive, especially for clinical application. DNA vaccines are much easier to produce, stable and easily stored [12].

In this study, we evaluated that the effects of APS as an adjuvant on HBV DNA vaccination in mice. Our results demonstrate for the first time that APS can efficiently enhance the immunogenicity of HBV DNA vaccines through improving DCs maturation and down-regulating the Treg frequency.

2. Materials and methods

2.1. Reagents and plasmids

APS for clinical application was purchased from Pharmagenesis Inc (Beijing, China) and was dissolved in the saline at the concentration of 50 mg/mL, then stored in −20 °C for further dilution. Fluorescent labeled

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anti-mouse monoclonal antibodies: CD8-PE,CD4-PE,IL-4-PE,CD4-FITC, IL-2-FITC, IFN-γ-FITC, CD11c-FITC, CD40-PE, CD80-PE and CD86-PE were purchased from eBiosciences (San Diego, CA). HBV recombinant plasmids encoding HBsAg(pcDS2) was constructed as described previously [13]. pcDS2 was prepared by alkali lysed maxi-preparation, and purified by PEG8000, and diluted the saline solution.

2.2. Animals and immunization

6–8-week-old female Balb/c mice were purchased from West China Laboratory Animal Center (Chengdu, China). All animals were housed and maintained in accordance with the Guide for the Care and Use of Laboratory Animals. 50 mice were randomly divided into 5 groups, namely, naïve group, pcDNA3 group, pcDS2 group, pcDS2 plus APS group and APS group. Immunization was performed on day 0, 14 and 28, with vehicles listed in Table 1.

2.3. Antibody ELISA

IgG concentration in serum was determined by ELISA on day 7 after the second boost immunization. The procedure was performed following the manufacture's protocols (SIIC Kinghaw Biotech Co., Ltd., Beijing, China) and the amount of total anti-HBsAg antibody was calculated as previously described [13].

2.4. T cell proliferation

Single splenocytes were prepared on day 7 after the second boost immunization, and suspended in RPMI 1640 (Hyclone, UT, USA) supplemented with 10% FBS (Hyclone, UT, USA). This method was performed according to the previously described protocols [13]. Proliferation level was determined using Cell counting Kit-8 (Beyotime, Haimen, China) according to the manufacturer's instructions. OD values at 450 nm were determined by a plate reader (Synergy HT, Bio-TEK). Data were expressed as the stimulation index (SI), calculated as the mean reading of triplicate wells stimulated with antigen, divided by the mean reading of triplicate wells stimulated with the medium.

2.5. Intracellular cytokine analysis

T cell suspension was prepared on day 7 after the third immunization. T cells at 1×10^6 cells/100 μl were stimulated in 96-well plates with HBsAg (5 μg/ml) or HBsAg-derived peptide S208–215 (ILSPFLPL; H-2K^b-restricted at 10 μg/ml) (GL Biochem, Shanghai, China) and anti-CD28 (5 μg/ml) mAb for 6 h at 37 °C and with 5% CO_2. Monensin (2 μg/ml) was added for the last 4 h and the cells were washed three times with PBS. Cells were blocked with Fcγ-Block (BD Phamingen, San Diego, USA) in PBS for 30 min at 4 °C before fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin, immunostained with isotype controls, or double stained with anti-CD4-FITC and anti-IL-4-PE, or anti-CD8-PE and anti-IFN-γ-FITC, or anti-CD4-PE and anti-IFN-γ-FITC, or anti-CD4-FITC and anti-IL-2-PE for 30 min at 4 °C. The cells were washed three times and then analyzed with a FACS Calibur using the Cell Quest Pro Software (BD Bioscience).

Table 1
Animal grouping.

Group	DNA vaccine	Adjuvant
1	Naive	
2	100 μg pcDNA3	
3	100 μg pcDS2	
4	100 μg pcDS2	500 μg APS
5		500 μg APS

2.6. In vivo CTL activity assay

In vivo cytotoxic assay was performed as described previously [13]. Briefly, the splenocytes from naïve Balb/c mice were divided into two equal portions. One portion was incubated with 10^{-6} M HBsAg CTL peptide S208–215, while the other was left intact. Then, the former and latter were labeled with a high concentration of CFSE (5 μM, CFSE^{high} cells) as target cells and a low concentration of CFSE (0.5 μM, CFSE^{low} cells) as control cells using the Live Cell Labeling Kit (Dojindo, Kumamoto, Japan). The target and control cells were mixed in a 1:1 ratio and injected into immunized mice at 2×10^7 cells per mouse via the tail vein on day 7 after the third immunization. Four hours later spleens of injected mice were isolated and the target and control cells were analyzed by their differential CFSE fluorescent intensities using a FACS Calibur (BD Biosciences, USA). Specific lysis was calculated using the following formula: ratio = percentage of CFSE low/percentage of CFSE high. Percentage of specific lysis = 1 − (ratio unprimed/ratio primed) × 100%.

2.7. RT-PCR

Total RNA was extracted from total splenocytes of the immunized mice with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was synthesized using Ace reverse transcriptase (Toyobo Co. Ltd, Pudong, Shanghai) with Oligo (dT) 18 primers (the primers for PCR are listed in Table 2). PCR products were resolved on 1.5% agarose gels and visualized by ethidium bromide staining under UV light.

2.8. DC surface co-stimulatory molecules staining

Mice were sacrificed on day 3 after the first immunization, single-cell suspensions from the spleens at 1×10^6 cells/100 μl were blocked with 1 μl of Fcγ mAb (0.5 μg/ml) for 30 min at 4 °C. After washed once with PBS, cells were used to stain with isotype controls, or double staining with anti-CD11c-FITC and anti-CD80-PE, or anti-CD11c-FITC and anti-CD86-PE, or anti-CD11c-FITC and anti-CD40-PE. The fluorescent intensities were measured by the FACS Calibur and analyzed by Cell Questpro software (BD Biosciences, USA).

2.9. Treg activity assay

Splenocytes were obtained on day 3 after the first immunization and stained using Mouse Regulatory T Cell Staining Kit (eBioscience, San Diego, CA) according to the manufacture's instruction. Briefly, 1×10^6 cells in 100 μL Flow Cytometry Staining Buffer were blocked with 1 μL anti-mouse CD16/CD32 antibodies for 20 min at 4 °C. After washing twice using PBS, cells were stained with anti-CD4-FITC, anti-CD25-APC for 39 min at 4 °C. Then 1 mL of Foxp3 Fixation/Permeabilization working solution was added and incubated for 1.5 h in room temperature. At last, anti-Foxp3-PE was used to stain the cells for 30 min. The cells were washed and analyzed with FACS Calibur using the Cell Quest Pro Software (BD Bioscience).

Table 2
Target gene primers.

Target gene	Sequence of primers
β-actin	F:5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′
	R:5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′
MHC I	F:5′-GGGAGACACAGAGAGCCAAG-3′
	R: 5′-GATGAGTCACATGGGCCTTT-3′
MHC II	F:5′-AGCCTCTGTGGAGGTGAAGA-3′
	R:5′-AAAGCAGATGAGGGTGTTGG-3′