Carbohydrate Polymers 110 (2014) 446–455

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Effect and mechanisms of curdlan sulfate on inhibiting HBV infection and acting as an HB vaccine adjuvant

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ARTICLE INFO

Article history:
Received 15 December 2013
Received in revised form 4 April 2014
Accepted 7 April 2014
Available online 20 April 2014

Keywords:
Curdlan sulfate
Anti-HBV
HBsAg
Th1
Vaccine adjuvant

ABSTRACT

In this study, the effect and mechanisms of curdlan sulfate (CS3) on hepatitis B virus (HBV) infection and promoting immune response of the mice immunized with recombinant hepatitis B surface protein (HBsAg) were investigated. The results showed that CS3 could inhibit HBV infection of HepG2 and HepaRG cells, especially the process of HBV particle binding to the cell surfaces. The surface plasmon response (SPR) technology indicated that CS3 could bind with recombinant HBsAg and the binding ability depended on the content of sulfate groups on the polysaccharide chains. Co-administration of CS3 to BALB/c mice immunized with HBsAg significantly enhanced the influx of macrophages and dendritic cells in spleen, increased antigen-specific $CD4^+$ and $CD8^+$ cell numbers, and promoted splenocyte proliferation. The titer of HBsAg-specific antibodies was also augmented by use of CS3 as a vaccine adjuvant. The higher expression of interferon (IFN)-γ, lower expression of interleukin (IL)-4, and higher IgG2a/IgG1 ratio within the anti-HBsAg antibodies in mice immunized with HBsAg plus CS3 than those in mice receiving HBsAg alone indicated that CS3 induced a shift toward a Th1-biased immune response. These results presented that CS3 could be developed as an immunotherapy agent or vaccine adjuvant for HBV infection treatment or prevention.

1. Introduction

Hepatitis B virus (HBV) infection, one of the most crucial chronic virus infections in the world, is considered as a leading factor of chronic liver diseases, cirrhosis, and primary hepatocellular carcinoma (HCC) (Yoffe & Noonan, 1992). Currently antiviral therapies involve the use of nucleoside analogs lamivudine and alpha-interferon (Pawlotsky, 2009). Although lamivudine is an effective anti-viral medicine (Lai et al., 1998), the long-term use may cause drug-resistant HBV mutants and viral breakthrough, and the occuring rate is 15–32% within the first year and jump to 67% after 4 years constant treatment (Lada et al., 2004). The alpha-interferon is effective in only about one third of naive patients, and it can induce dose-limiting adverse effects during the therapy (Mutimer et al., 1998; Wong et al., 1993). Therefore, new treatment strategies or novel medicines are urgently needed in the HBV infection treatment.

The pathogenesis of HBV infection associated diseases is not directly cytopathic to hepatocytes, but a complex interaction between the virus and the host immune response (Ratnam & Visvanathan, 2008). Studies proved that viral hepatitis is initiated by an antigen-specific antiviral cellular immune response, and the clearance of virus infections needs the killing of infected cells by virus-specific T cells, as well as the intracellular viral inactivation by certain inflammatory cytokines released by activated lymphomononuclear cells (Chisari, 2000). However, the clearance of HBV is difficult because HBV can escape from host innate and adaptive immunity, and induce the immune tolerance. The immunosuppressed environment of HBV-caused immune tolerance in the liver including functional impairment of dendritic cells

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http://dx.doi.org/10.1016/j.carbpol.2014.04.025
0144-8617/

(DCs), T cells, NK cells, imbalance of cytokines, and so on (Han, Zhang, Zhang, & Tian, 2011). Thus, the therapeutic strategy of HBV infection aimed at modulating the host immune activity may represent a complementary approach to antiviral treatment (Wang & Zhang, 2009).

So far, the most effective way to prevent susceptible people from virus infection is to immunize them with hepatitis B (HB) vaccines, with approximately 90–95% protective efficacy. The acute and chronic infection rates, the mortality of fulminant hepatitis B in infants and the incidence of hepatocellular carcinoma have been effectively reduced by about 25% since the development of HB vaccine. The HB vaccine, both early plasma-derived and latter recombinant DNA-derived, contains one of the viral envelope proteins, hepatitis B surface antigen (HBsAg), which plays an important role in viral immunity (Vildozola, 2007). However, nearly 5–10% of immunized individuals do not respond adequately to the vaccine and remain at risk for hepatitis B infection (Goncalves et al., 2006). At present, alum (aluminum hydroxide) remains the only adjuvant for HB vaccine approved for human use in USA, but unfortunately, it has no effect on cellular immunity (Khajuria et al., 2007). Therefore, to enhance the immunogenicity of HB vaccine, adjuvants or potentiators need to be developed. Novel adjuvant and formulations will be required to improve the cell mediated immune response to recombinant antigens.

Sulfated polysaccharides are generally considered to have varieties of biological activities, such as anti-tumor, anti-virus, anti-inflammation, immunomodulating and anti-coagulation (Li, Sheng, et al., 2013; Li, Zhang, et al., 2013; Zong et al., 2013). Curdlan is an extracellular polysaccharide produced by the bacteria of *Alcaligenes faecalis* var. *myxogenes* 10C3, mutant K, with the structure of 1,3-β-D-glucans with no glycosyl side chains (Harada, Masaki, & Saito, 1968). Its sulfated derivatives, curdlan sulfate, has been studied as an anti-HIV agent since 1990, and the study has carried out in clinical phase II with good efficiency and low toxicity (Gordon et al., 1997). Our previous study (Li, Sheng, et al., 2013; Li, Zhang, et al., 2013) found that curdlan sulfate possessed immunomodulating activities, such as inducing mouse splenocytes proliferation, macrophages activation, as well as bone marrow derived dendritic cells (BMDCs) maturation, and the activities were realized probably through the primary receptor dectin-1 molecule on the cell surface. Based on these results, the present study was to validate our hypothesis that curdlan sulfate may act as a potential therapeutic agent possessing anti-HBV and immunoadjuvant activities.

2. Materials and methods

2.1. Reagents and antibodies

Curdlan sulfates with different sulfur content were prepared as described previously (Li, Sheng, et al., 2013; Li, Zhang, et al., 2013). CS3, curdlan sulfate with the sulfur content of (9.23 ± 0.16)%, was used in activity assays. It was dissolved in serum-free medium for the *in vitro* assay and normal saline (NS) for the *in vivo* assay. Heparin sodium, MW 15.33 kDa and MW 19.00 kDa were obtained from SaiNuoKang Biochemical Co., Ltd. (Zaozhuang, China) and TianDong Pharmaceutical Co., Ltd. (Dongying, China), respectively. Chondroitin sulfate (MW 28.40 kDa) was from KangPing Bio-Tech Co., Ltd. (Linyi, China). The antibodies, anti-mouse F4/80 antigen PE (clone BM8), anti-mouse CD40 FITC (clone HM40-3), anti-mouse MHC class II (I-A/I-E) FITC (clone M5/114.15.2), anti-mouse CD11c PE (clone N418), anti-mouse CD86 FITC (clone GL1), anti-mouse CD4 FITC (clone GK1.5), anti-mouse CD8a PE (clone 53–6.7), anti-mouse CD69 PE (clone H1.2F3), anti-mouse IL-4 PE (clone 11B11), and anti-mouse IFN-γ APC (clone XMG1.2), were all purchased from eBioscience (Boston, MA, USA). The recombinant HBsAg, and the commercial HB vaccine were both obtained from Dalian Hissen Bio-pharm Co., Ltd. (Dalian, China). HBsAg and HBeAg ELISA kits were purchased from RongSheng Biological Pharmaceutical Co., Ltd. (Shanghai, China). The anti-HBsAg ELISA kit was obtained from LanTu Bio-Tech Co., Ltd. (Quanzhou, China). Horseradish peroxidase (HRP) conjugated antibody against IgG1 or IgG2a were purchased from Proteintech Group, Inc (Chicago, USA). All other chemicals and reagents used were analytical grade.

2.2. Cell lines and animals

HepG2.2.15 cells (serotype ayw, genotype D), derived from HepG2 cells transfected with a plasmid carrying two head-to-tail copies of HBV genome DNA, were maintained in complete Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), and 400 μg/mL G418 (Invitrogen). HepG2 (maintained in our lab) were cultured in DMEM medium supplemented with 10% FBS, and penicillin–streptomycin (100 IU/mL–100 μg/mL). Differentiate HepaRG cells, a hepatoma-derived cell lines that expresses a representative panel of liver-specific genes and is susceptible to HBV infection (Gripon et al., 2002), was maintained in William's E medium supplemented with 10% FBS, penicillin–streptomycin (100 IU/mL–100 μg/mL), 5 μg/mL insulin, and 5×10^{-5} M hydrocortisone hemisuccinate (Sigma–Aldrich).

Wide-type female BALB/c mice (5–9 weeks) were obtained from Beijing HFK Bioscience Co., LTD (Beijing, China), and housed under pathogen-free conditions. The use of animals was approved by the Institutional Animal Care Committee of Shandong University, with firm adherence to the Ethical Guidelines for the Care and Use of Animals.

2.3. Anti-HBV infection assay

2.3.1. Preparation of the hepatitis B virus inoculum

The HBV particle was prepared from an approximately 100-fold concentrated culture supernatant of HepG2.2.15 cells because of an unlimited supply and a constant quality (Gripon et al., 2002). Briefly, it was prepared from freshly collected supernatants by precipitating viral particles in the presence of 10% polyethylene glycol (PEG) 8000 (Merck) at 4 °C overnight. Then the solution was centrifuged at 8000 rpm for 30 min at 4 °C. The pellet was resuspended in PBS containing 25% FBS. Aliquots (100 μL) were stored at −80 °C. When used, the solution containing HBV particles was further centrifuged at 10,000 rpm for 30 min at 4 °C, and the supernatant was used to infect cells.

2.3.2. Measurement of the compounds or HBV particles acting on HepaRG and HepG2 cells proliferation

To test whether curdlan sulfate, and heparin sodium (MW 15.33 kDa) influence the proliferation of the differentiated HepaRG and HepG2 cells, the cells were seeded in a 96-well plate at a density of 3×10^4 cells/mL in 100 μL medium with different final concentrations of curdlan sulfate and heparin sodium (20, 100, 500 μg/mL, dissolved in serum-free medium) for 24 h at 37 °C and 5% CO_2. Twenty microliter of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) solution (5 mg/mL in phosphate buffered-saline) was added to each well 4 h prior to culture termination. Then the supernatant was discarded and 150 μL of DMSO was added. After shaking to dissolve the formazan, the optical density (OD) at 570 nm was measured by a microplate reader (Bio-Rad). Each experimental condition was tested in triplicate.

The HBV particles prepared as mentioned above was used to co-culture with HepaRG or HepG2 cells. The cells were cultured at a density of 3×10^4 cells/mL in 100 μL medium, and the supernatant

containing HBV particles were inoculated with the final concentration of 12.5% (v/v) and 25% (v/v) for 24 h at 37 °C and 5% CO_2. MTT assay was applied to test the proliferation of cells as described above.

2.3.3. HBV binding and infection of HepG2 and HepaRG cells

Differentiated HepaRG cells or HepG2 cells were seeded in a 6-well plate at a density of 5×10^5 cells/well, 2 mL for each well. Two days after plating, the cells were inoculated with 500 μL of HBV particle stock as well as the corresponding tested compounds with different concentrations or medium in the presence of PEG 8000 with a final concentration of 4%. The binding assay was performed at 37 °C for 4 h, and then the inoculum was removed (Schulze, Gripon, & Urban, 2007). The cells were then extensively washed with warm phosphate-buffered saline (PBS) for five times gently, and then harvested for DNA extraction. For the infection assay, the cells were incubated with HBV and tested compounds for 24 h at 37 °C, then discarded the HBV inoculum and cultivated further for measuring secretion of newly synthesized HBsAg and HBeAg (hepatitis B e antigen). All experiments were performed in duplicates and at least 2 times independently.

2.3.3.1. Quantitative real-time PCR analysis of HBV DNA. The HepaRG or HepG2 cell DNA was extracted using the commercial kit (Tiangen Biotech, Co., LTD. Beijing, China), and the viral DNA was quantified by real-time PCR according to the instructions of the FQ-PCR HBV-DNA kit (Da-An, Guangzhou, China). The primer sequences specific for HBV S region were: upstream 5′ ATCCTGCTGCTATGCCTCATCTT 3′, and downstream 5′ ACAGGGGGAAAGCCCTACGAA 3′. The fluorescent probe was 5′ FAM-TGGCTAGTTTACAGTGCCATTT GTAMRA 3′, with FAM as the reporter fluorophore, and TAMRA as the quencher fluorophore. The reaction was carried out for 42 cycles in the Roche LightCycler 480 real-time PCR system (Han et al., 2011).

2.3.3.2. HBsAg and HBeAg detection by ELISA. Cells were cultivated and the medium was changed every 2 days. To quantify the infection, HBsAg and HBeAg secreted into the culture supernatant were collected at day 7 and detected using ELISA kits from RongSheng (Shanghai, China).

2.4. Affinity of recombinant HBsAg to curdlan sulfates, heparin sodium and chondroitin sulfate

The affinities of curdlan sulfates, heparin sodium and chondroitin sulfate to HBsAg were examined by biomolecular interaction analysis (BIA). SPR-based measurements were performed at 25 °C with a BIAcore 3000 instrument (GE Healthcare, Sweden) according to manufacturer's instruction, using the running buffer of HBSEP (10 mM HEPES buffer (pH 7.4), containing 150 mM NaCl, 3 mM EDTA and 0.005% Surfactant P20). Before analysis, recombinant HBsAg in sodium acetate solution (10 mM, pH 3.7) was covalently attached to CM5 biosensor chips using a standard amine coupling kit (GE Healthcare). Different concentrations of curdlan sulfates, heparin sodium, and chondroitin sulfate (1 000, 500, 250, 125, 62.5, 0 μg/mL in the running buffer; 250 μg/mL was used as the repeated concentration) were injected into channels to record the signals. All signals were recorded as sensorgrams. After each injection cycle, surfaces were regenerated with one injection of 15 μL of 10 mM glycine-HCl (pH 2.5). In the kinetics analysis, global fit analysis was performed using a BIA evaluation 4.1 software, rate equations derived from the 1:1 Langmuir binding model were fitted to the experimentally obtained association and dissociation phases binding curves for all injections. The equilibrium dissociation constant (K_D) value was calculated by the software automatically.

2.5. Immunization of mice with HB vaccine, HBsAg and curdlan sulfate

The immunization assay was performed as follows: BALB/c mice were divided into seven groups, and were immunized subcutaneously with normal saline, 5 μg HBsAg alone, HB vaccine (alum as the adjuvant), 5 μg HBsAg with 0.2 mg CS3, 0.5 mg CS3, 1.0 mg CS3, and 1.0 mg curdlan, in 0.1 mL normal saline, respectively. Immunization was performed on day 0, 14, 28 with the same treatment schedule.

2.5.1. Spleen lymphocyte preparation

At day 35 after the HBsAg primary immunization, splenocytes were collected from three mice/group, subjected to treatment with lysis buffer (139.6 mM NH_4Cl, 21.5 mM Tris, pH 7.2) to eliminate red cells, washed with Hank's balanced salt solution (HBSS, Solarbio, Beijing, China) buffer and resuspended in complete medium (RPMI-1640 medium supplemented with 10% FBS and penicillin–streptomycin).

2.5.2. Flow cytometric analysis of surface markers

Flow cytometer (FACS calibur, Becton Dickinson) was used to determine the surface expression of CD3, CD4, CD8 and F4/80 molecules on spleen leukocytes. Co-expression of CD11c with CD40, CD86, and MHC class II were also tested, as well as co-expression of CD4 with CD69. In brief, the cells were stained with relevant fluorochrome-labeled mAbs, washed with PBS buffer, and then sent to FACScan flow cytometer. Fluorescence profiles were generated, and dot plot was produced by the WinMDI29 software.

2.5.3. Spleen lymphocyte proliferation assay

Splenocytes were collected from the HBsAg-immunized mice under aseptic conditions. Cell numbers were counted using a haemocytometer by trypan blue dye exclusion technique, and the cell viability exceeded 95%. The cells were seeded into three wells of a 96-well flat-bottom microtiter plate at 2×10^6 cells/mL in 100 μL complete medium, thereafter HBsAg with the final concentration of 0.5 μg/mL, or medium was added giving a final volume of 200 μL. After the plates were incubated at 37 °C in a humid atmosphere with 5% CO_2 for 72 h, MTT method was used to test the proliferation of splenocytes as mentioned in Section 2.3.2.

2.5.4. Cytokines measurement by ELISA

Splenocytes were seeded into three wells of a 96-well plate at 2×10^7 cells/mL in 100 μL complete medium. The cells were incubated with or without HBsAg (0.5 μg/mL) for 72 h at 37 °C and 5% CO_2. The culture supernatants were harvested for the detection of cytokines including IFN-γ and IL-4 by ELISA, according to the protocol of manufacturer.

2.5.5. Detection of intracellular cytokines by flow cytometry

Seven weeks after primary immunization, IL-4 and IFN-γ production were determined by intracellular cytokine staining. Splenocytes were collected from three mice/group under aseptic conditions, and incubated at a density of 2×10^7 cells/well in 24-well plates with or without HBsAg (0.5 μg/mL) for antigen-specific re-stimulation for 24 h. Then cells were stimulated with the cell stimulation PMA (20 ng/mL) plus ionomycin (1 μg/mL) (Sigma–Aldrich) for 4 h in the presence of Brefeldin A (10 μM) and Monensin (2 μM) (protein transport inhibition cocktail, eBioscience). After incubation, the cells were washed with PBS and then stained with anti-CD8 and anti-CD4 mAbs at 4 °C for 1 h, and

then treated with Fix&Perm cell permeabilization reagents (Multi-Sciences, Biotech Co., Ltd, Shanghai, China) and washed with PBS. The cells were stained with PE-IL-4 and APC-IFN-γ at 4 °C for 2 h and analyzed by flow cytometry.

2.5.6. Detection of anti-HBsAg antibodies and immunoglobulin subtypes production in mice sera

Blood was collected from the retroorbital plexus of mice under ether anesthesia and the serum was prepared at 7, 21, and 35 days after the primary immunization. The titer of anti-HBsAg antibodies was determined by anti-HBs EIA kit (LanTu Bio-Tech), following manufacture's instructions. The HBsAg-specific IgG1 and IgG2a antibodies in serum were detected by an indirect ELISA. In brief, 50 μL of five-fold diluted serum samples was added to each microtiter plate wells coated with HBsAg (LanTu Bio-Tech). Next, the horseradish peroxidase (HRP) conjugated antibody against IgG1 or IgG2a was added to the wells, and incubated for 60 min at 37 °C. After five times of washing, 50 μL of substrate solution was added to each well followed by incubation for 15 min at 37 °C, and then the reaction was terminated by adding 50 μL/well of stop solution. The OD was determined using microplate reader at 450 nm.

2.6. Statistical analysis

Data are expressed as mean ± SEM, and the statistical significance between various groups was analyzed by Student's *t*-test one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered to be statistically significant. Statistical analysis was done using SPSS 17.0 software.

3. Results and discussion

3.1. CS3 can inhibit HBV infection in vitro

The HBV infects hepatocytes by a complex process with a still poorly understood mechanism, especially the attachment and entry processes. Studies have found that the glycosaminoglycans expressed on the cell surface, such as heparan sulfate proteoglycans, acts as a low-affinity receptor mediating the initial interaction of HBV with hepatocytes (Ying et al., 2002). Highly sulfated polysaccharides, including heparin sodium and dextran sulfate, can inhibit HBV infection of primary human hepatocytes, HepG2 and HepaRG cells (Kao & Chen, 2002; Schulze et al., 2007; Ying et al., 2002), and they were supposed to interrupt with the virus binding with host cells surface. Therefore, our study was to found out whether CS3 could also inhibit HBV infection *in vitro* using heparin sodium as a control.

3.1.1. CS3, heparin sodium or HBV particles did not influence the cells proliferation

It was necessary to evaluate whether the tested compounds or HBV particles have cytotoxic effect or proliferation stimulating effect on HepG2 and HepaRG cells before further tests. MTT assays indicated that neither CS3 nor heparin sodium showed cytotoxic or stimulating effect on cells in 24 h under the tested concentrations (Fig. 1A). And also, the co-culture of cells with HBV particles for 48 h did not influence the proliferation of HepG2 or HepaRG cells, as shown in Fig. 1B. These results exclude the possibility that the anti-HBV infection effect of curdlan sulfate and heparin sodium *in vitro* may be due to the growth inhibition on cells.

3.1.2. CS3 and heparin sodium inhibited HBV binding and infection of HepG2 and HepaRG cells

To test whether the sulfated polysaccharides, CS3 and heparin sodium, inhibit HBV binding and infection processes, we performed competition inhibition experiments in the presence of different concentrations of the compounds. The results of FQ-PCR analysis indicated that both CS3 and heparin sodium could decrease the HBV DNA content of the infected cells, HepG2 and HepaRG cells as shown in Fig. 1C and D, respectively. Heparin sodium exhibited an inhibition potential of 95% at the tested concentrations, with no apparent dose-dependent manner. At higher concentrations (100 and 500 μg/mL), CS3 could inhibit HBV infecting HepG2 and HepaRG cells significantly ($^{**}p < 0.01$), but showed weaker effect than heparin sodium.

The levels of HBsAg and HBeAg secreted into the medium by infected cells at day 7 were determined to evaluate the infection inhibition effect of CS3 and heparin sodium. As shown in Fig. 1E–H, both CS3 and heparin could inhibit the secretion of HBsAg and HBeAg in a dose-dependent manner. CS3 showed significantly inhibitory effect on HBV infecting HepG2 and HepaRG cells at the concentration of 500 μg/mL compared with the uncompeted control infection ($^{*}p < 0.05$, $^{**}p < 0.01$). Heparin also abolished HBV infection of the two cell lines significantly at the tested concentrations of 100 μg/mL (HBsAg) and 500 μg/mL (HBsAg and HBeAg) ($^{*}p < 0.05$, $^{**}p < 0.01$).

The FQ-PCR and ELISA results indicated that CS3 and heparin have the ability of inhibiting HBV binding and infection of the susceptible cell lines of HepG2 and HepaRG. Heparin has been reported to interfere with the binding and infection processes of HBV in previous studies (Leistner, Gruen-Bernhard, & Glebe, 2008; Schulze et al., 2007; Ying et al., 2002), which is proved dominantly due to the sulfation groups of the structure, and the possible mechanism is that heparin acts as an competitor binding with HBV to interfere the interaction between HBV and one of its receptors on the cell membrane, and we think CS3 may act in the same way.

Heparan sulfate (HS) is one kind of sulfated glycosaminoglycans, covalently connected with a core protein to form the HS proteoglycans (HSPGs), which can mediate the virus binding or entry into the host cells (Li, Sheng, et al., 2013; Li, Zhang, et al., 2013). Studies have proved that HBV infection of hepatocytes uses HSPGs as low-affinity receptor, and HBV infection can be inhibited by heparin and other high-sulfated polymers, by interfering with the binding of HBV surface protein (HBsAg) to HSPGs (Leistner, Gruen-Bernhard, & Glebe, 2008; Schulze et al., 2007). Further studies found that two positively charged residues R122 and K141 positioned in an antigenic loop in the HBV envelope proteins could bind with HS through electrostatic interactions (Sureau & Salisse, 2013). Therefore, we suspect that the reason why CS3 showed weaker inhibitory effect than heparin sodium is mainly due to the lower sulfur content than that of heparin sodium, that is, lower sulfur content, weaker binding affinity with HBV particles. Therefore, we applied SPR technology to find out the binding affinities of HBsAg to polysaccharides with different sulfur content.

3.2. CS3 can bind with recombinant HBsAg

SPR technology has been used to characterize the interactions between antibody–antigen, ligand–receptor, and proteins with carbohydrate for years (Myszka, 1997). Here we applied SPR with a BIAcore 3000 biosensor system to analyze the affinity of different sulfated polysaccharides to recombinant human HBsAg. Experiments at 25 °C showed that HBsAg was immobilized on the CM5 chip with a coupling capacity of approximately 10 000 RU (data not shown). K_D (equilibrium dissociation constant) value is an important parameter for the measurement of ligand–receptor interactions and the smaller the value, the greater the binding capacity. All the sensorgrams and K_D values were listed in Fig. 2 and Table 1. Curdlan sulfates with different sulfur content showed different binding affinities toward HBsAg. CS3 with the highest sulfur content (9.23 ± 0.16%) can bind to HBsAg with a K_D value of $(1.72 \pm 3.43) \times 10^{-7}$, which is much smaller than that of CS2 (sulfur

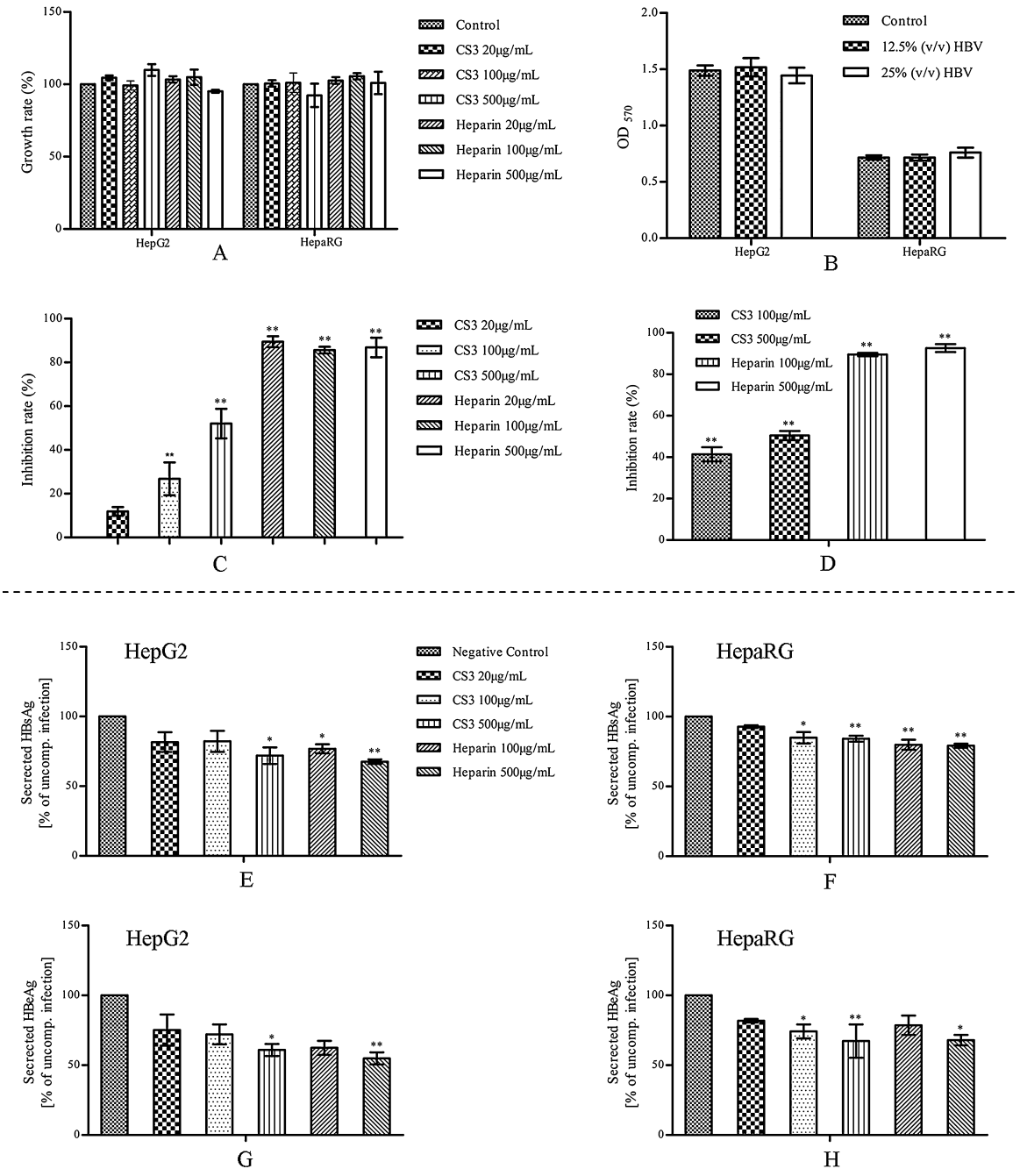

Fig. 1. Effect of CS3 and heparin sodium on HBV infecting of HepG2 and HepaRG cells. (A) Effect of CS3 and heparin sodium on the proliferation of HepG2 and HepaRG cells after treatment for 24 h. (B) Effect of HBV particles on the proliferation of HepG2 and HepaRG cells after treatment for 48 h. (C) and (D) Effects of CS3 and heparin sodium on the HBV DNA content of the virus bound to HepG2 and HepaRG cells, respectively. $^{**}p < 0.01$, compared with control group ($n = 3$). (E) and (F) Effect of CS3 and heparin sodium on the secretion of HBsAg and HBeAg on day 7 after the cells infected with HBV. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with uncompeted infection negative control ($n = 3$).

content: 6.27 ± 0.03%) and CS1 (sulfur content: 4.03 ± 0.3%), indicating that CS3 has the highest affinity to HBsAg, whereas CS1 has the lowest binding affinity. Heparin sodium and chondritin sulfate tested in this assay exhibited the similar results, *i.e.*, heparin sodium with the higher sulfur content (10.51 ± 0.10%) had a lower K_D value than the one with the lower sulfur content (9.49 ± 0.09%). Chondroitin sulfate could hardly bind to HBsAg, and this is in accordance with the result of anti-HBV infection study *in vitro* carried out in our

Table 1
The sulfated polysaccharides with different molecular weight, sulfur (S) content, and the binding affinity K_D (equilibrium dissociation constants) values of the polysaccharides with recombinant HBsAg.

Sample	CS1	CS2	CS3	Heparin sodium	Heparin sodium	Chondritin sulfate
MW (kDa)	25.87	87.03	171.90	19.00	15.33	28.40
S content (%)	4.03 ± 0.3	6.27 ± 0.03	9.23 ± 0.16	9.49 ± 0.09	10.51 ± 0.10	4.56 ± 0.06
K_D (M)	$(1.37 \pm 2.38) \times 10^{-5}$	$(3.16 \pm 5.48) \times 10^{-6}$	$(1.72 \pm 3.43) \times 10^{-7}$	$(4.06 \pm 4.96) \times 10^{-7}$	$(2.28 \pm 1.41) \times 10^{-9}$	$(1.80 \pm 3.43) \times 10^{-4}$

The value represents mean ± SD ($n = 3$).

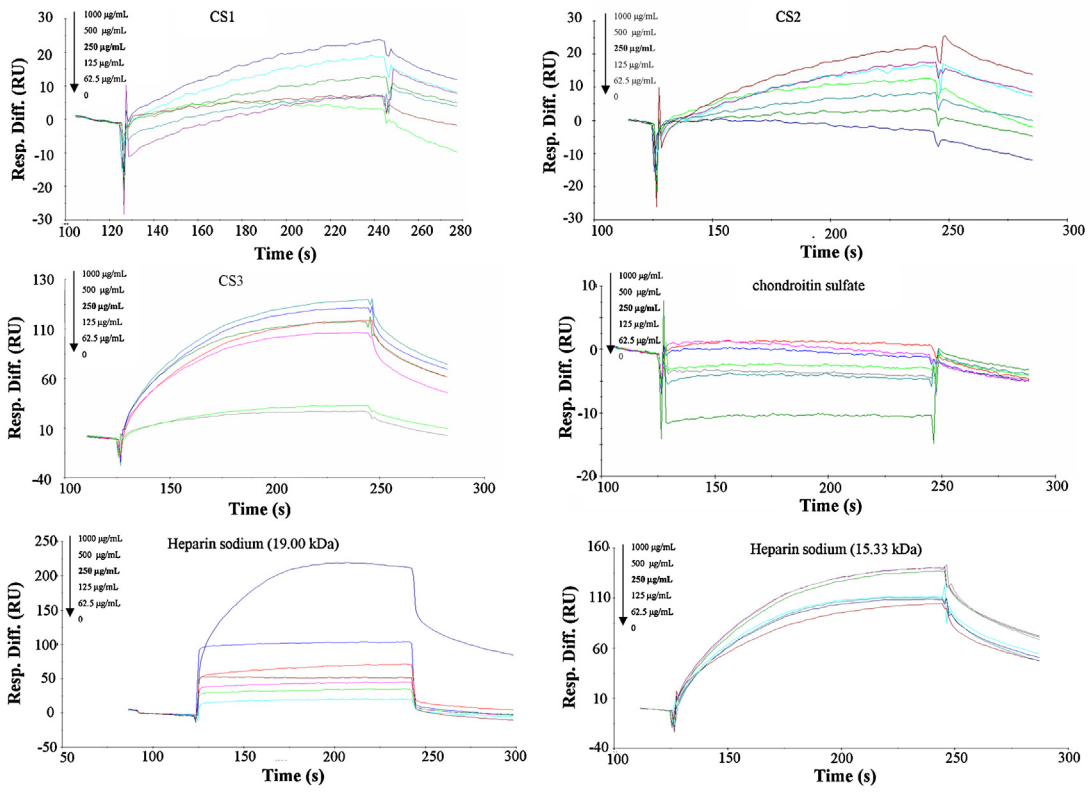

Fig. 2. Binding affinities of different sulfated polysaccharides against recombinant HBsAg protein evaluated by SPR. Six serial dilution concentrations of the compounds were injected at 30 μL/min for 60 s association periods. The concentrations were shown next to the arrows from up to down. The bold figures, 250 μg/mL was used as the repeated concentration. The sensorgrams were obtained and the K_D values were analyzed using a global fit algorithm (BIA evaluation 3.1).

assay (data not shown). The SPR analysis results indicated that the anti-viral infection mechanism of sulfated polysaccharide may due to the polyanions structure in some extent, and the degree of sulfation on the carbohydrate chains also plays a critical role in its anti-HBV infection effect.

3.3. CS3 can enhance the immune response of the mice immunized with HBsAg

Polysaccharides from various origins (fungi, bacteria, plants, *etc.*) possess important immunomodulatory effects, especially in activating of macrophages and DCs through selective receptors, such as toll-like receptor and dectin-1. The polysaccharides mainly composed of 1,3-β-D-glucans are thought to be more active in stimulating immune effect than 1,6-linkages (Dong et al., 2007). Studies have demonstrated that some polysaccharides could enhance the immune response and act as an effective adjuvant for vaccines (Dong et al., 2007; Huang et al., 2008; Wang et al., 2010). Therefore, we studied the effects of CS3 on the immune response of mice immunized with HBsAg, to investigate the potential activity of CS3 as a vaccine adjuvant and immunotherapy agent for HBV infection prevention and treatment.

3.3.1. Effect of CS3 on HBsAg-induced recruitment of macrophages and maturation of dendritic cells in the spleen

CS3 has been evidenced to enhance both humoral and cellular immunity by binding to specific receptor dectin-1 expressed on monocytes and other antigen-presenting cells (APCs), resulting in the activation of NF-κB, maturation of monocyte and expression of proinflammatory cytokines *in vitro* (Li, Sheng, et al., 2013; Li, Zhang, et al., 2013). Activated macrophages and BMDC are important antigen-presenting cells (APCs) regulating innate and adaptive immune responses during microbial infection. BALB/c mice were immunized with HBsAg and corresponding compounds subcutaneously at day 0 and boosted at day 14 and 28. Compared to normal, co-administration of CS3 or curdlan at the concentration of 1.0 mg/mice induced a significant influx of F4/80$^+$ cells in spleen ($^{**}p < 0.01$), while no apparent effect on the macrophage number appeared with the administration of HBsAg alone or HB vaccine (Fig. 3A). Notably, the recruitment of macrophages in the spleen of polysaccharide-treated mice with high dosage were higher than that in the animals receiving the antigen alone or HB vaccine ($^{\#\#}p < 0.01$, compared with HBsAg alone; $^{++}p < 0.01$, compared with HB vaccine).

The transferring of dendritic cells to the central lymphoid organ and their HBsAg-drived maturation have also been studied in the immunized mice. The influx of CD11c$^+$ cells were stimulated at day 35 after immunization, however, no significant increase was observed in HBsAg and HB vaccine treated mice. Only CS3 in high dosage greatly induced the migration of DCs to the spleen in response to HBsAg. The same results were obtained when evaluating the expression of maturation markers of the class II MHC molecules (MHC II), CD40, and CD86 (Fig. 3B). Studies have found that monocyte-derived DCs from patients with chronic hepatitis B (CHB) infection were functionally impaired, which may reduce the antigen-presenting capacity of DCs and depress T help cell type 1 (Th1) response (Beckebaum et al., 2003). CS3 could increase the number and maturation of antigen-specific DCs in spleen, and may play an important role in enhancing immune responses to HBV.

3.3.2. Effect of curdlan sulfate on spleen T-cell subtypes

Splenocytes were isolated from mice immunized with HBsAg and the percentage of CD4$^+$ and CD8$^+$ T-cell subpopulations were examined by flow cytometric analysis. As shown in Table 2, CS3 and curdlan could significantly amplify the HBsAg-induced recruitment

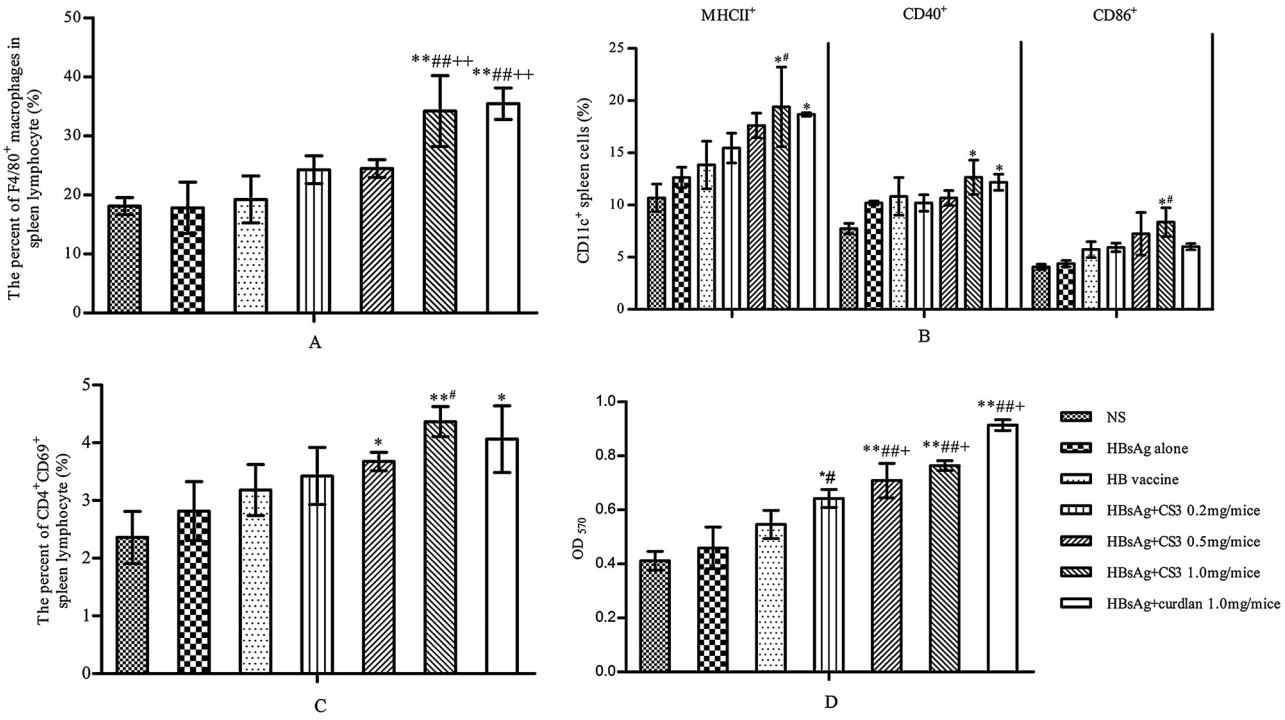

Fig. 3. Effect of CS3 on the immune response of the mice immunized with HBsAg. (A) Effect of CS3 on HBsAg-induced recruitment of macrophages in the spleen. (B) Effect of CS3 on HBsAg-induced influx and maturation of DCs in the spleen. The up-regulated expressions of MHCII, CD40, and CD86 represent the maturation of DCs. (C) Effect of CS3 on the activation of antigen-specific CD4 cells. (D) Evaluation on the proliferation of splenocytes from mice immunized with HBsAg plus or not adjuvant 35 days after the primary immunization under re-stimulation with HBsAg for 72 h. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with NS group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with HBsAg alone group; $^{+}p < 0.05$, $^{++}p < 0.01$, compared with HB vaccine group ($n = 3$).

of $CD3^+CD4^+$ T cells in the spleen, as well as that of $CD3^+CD8^+$ T cells, compared to NS control, HBsAg, and HB vaccine groups. The number of activated $CD4^+$ T cells in the spleen was also assessed by means of expression of the activation marker CD69. At day 35 after priming immunization, the number of $CD4^+CD69^+$ T cells was about 1.5-fold higher than that in the mice immunized with HBsAg alone (Fig. 3C, $^{\#}p < 0.05$ *vs.* HBsAg alone group).

3.3.3. *Effect of CS3 on splenocytes proliferation in HBsAg immunized mice*

The effect of CS3 on HBsAg-stimulated splenocytes proliferation in mice immunized with HBsAg is shown in Fig. 3D. The splenocytes proliferation in the mice immunized with CS3 and curdlan were significantly higher than that in the HBsAg and HB vaccine groups in a dose-dependent manner. Neither HBsAg nor HB vaccine treatment influenced the splenocytes proliferation in our assay. It was reported that in CHB patients, the antiviral T-cell responses including IFN-γ and T-cell responses were both down-regulated (Maini et al., 2000). Our results found that CS3 and curdlan could enhance the proliferative capacity of HBsAg stimulated splenocytes.

Table 2
The percentage of CD4 and CD8 subtype T cells in splenocytes.

Treatment	Portion of positive spleen cells (%)	
	$CD3^+CD4^+$	$CD3^+CD8^+$
Normal saline	15.85 ± 3.20	5.38 ± 0.96
HBsAg alone	18.94 ± 1.51	5.71 ± 0.94
HB vaccine	20.93 ± 3.84	6.23 ± 1.55
HBsAg + CS3 0.2 mg/mice	23.70 ± 1.27*	8.20 ± 0.28*
HBsAg + CS3 0.5 mg/mice	24.28 ± 3.28**	8.53 ± 1.17$^{*,\#,+}$
HBsAg + CS3 1.0 mg/mice	29.95 ± 0.50$^{**,\#\#,+}$	10.82 ± 1.50$^{**,\#\#,++}$
HBsAg + curdlan 1.0 mg/mice	25.45 ± 5.85$^{**,\#}$	7.80 ± 3.49*

Each value represents the mean ± SEM ($n = 3$).
* Compared with normal saline group, $p < 0.05$.
** Compared with normal saline group, $p < 0.01$.
$^{\#}$ Comapred with HBsAg alone group, $p < 0.05$.
$^{\#\#}$ Comapred with HBsAg alone group, $p < 0.01$.
$^{+}$ Comapred with HB vaccine group, $p < 0.05$.
$^{++}$ Comapred with HB vaccine group, $p < 0.01$.

3.3.4. *Effect of CS3 on Th1/Th2 cytokines in HBsAg immunized mice*

The mice splenocytes from different groups were obtained in the aseptic environment, and further cultured for 3 days with or without the HBsAg re-stimulation. Cytokine expressions in the supernatant were detected by ELISA kit to test the effect of CS3 on HBsAg-specific T helper (Th) cell response, while IFN-γ represents Th1 type, and IL-4 indicates Th2 response (Dong et al., 2007). As shown in Fig. 4A, CS3 at the dosage of 0.5 mg/mice could increase the IFN-γ expression significantly compared with NS. The IFN-γ expression both in CS3 and curdlan at the dosage of 1.0 mg/mice were markedly higher than that in HB vaccine group. The high expression of IFN-γ indicates that CS3 mixed with HBsAg was apt to enhance the cell immune response to antigens by Th1 cells. The effect of CS3 acting on IL-4 secretion was strange: at the dosage of 1 mg/mice, CS3 could increase the expression of IL-4 significantly, but at lower concentration, it inhibited the antigen induced IL-4 expression dose-dependently, compared with HBsAg alone and HB vaccine groups (Fig. 4B). Curdlan at the tested concentration also decreased the secretion of HBsAg stimulated IL-4. CS3 at lower concentrations as well as curdlan may suppress the antigen induced Th2 type in some extent, and this is accordance with the previous study that curdlan could inhibit OVA-induced Th2 cytokine production in the airways (Kawashima et al., 2012). However, CS3 turned to enhance the humoral response to HBsAg by Th2 cells in some extent when the concentration reached 1.0 mg/mice, which need to be studied further. Overall, the effect of CS3 on up-regulating the

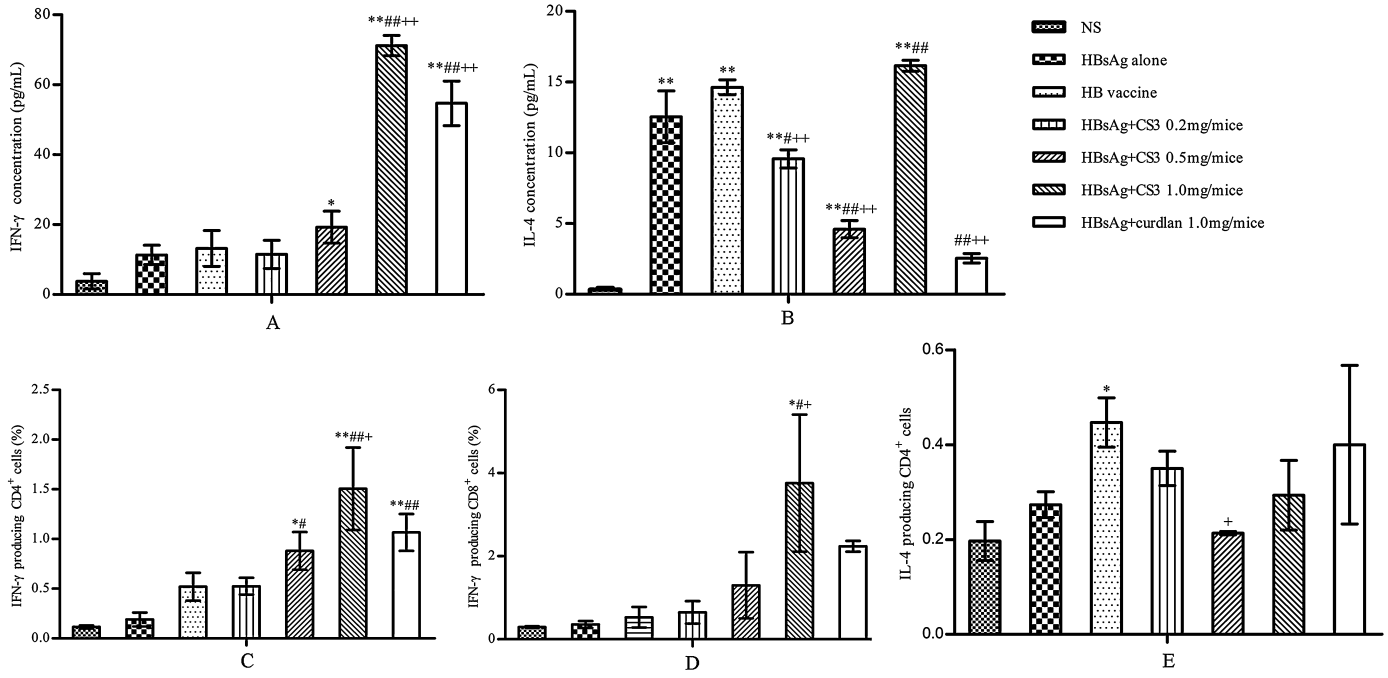

Fig. 4. Effect of CS3 on the expression of HBsAg-specific Th1/Th2 cytokines. IFN-γ and IL-4 were studied as Th1 and Th2 type cytokines, respectively. (A) and (B) The secretions of IFN-γ and IL-4 in the supernatant of cultured splenocytes re-stimulated with HBsAg for 72 h. (C)–(E) The IFN-γ-producing CD4 and CD8 cells, and the IL-4-producing CD4 cells detected by intracellular stain method. $^{*}p<0.05$, $^{**}p<0.01$, compared with NS group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared with HBsAg alone group; $^{+}p<0.05$, $^{++}p<0.01$, compared with HB vaccine group ($n=3$).

expression of IFN-γ was more notable than that of IL-4, and CS3 may activate Th1 type immune response to HBsAg immunization.

3.3.5. *Effect of CS3 on the levels of intracellular cytokines*

To further confirm the immune type of CS3, we also stained the splenocytes to assess the intracellular cytokines contents. The similar high expression of intracellular IFN-γ was observed in cultured splenocytes with HBsAg re-stimulation from the group of CS3 at the dosage of 1.0 mg/mice (Fig. 4C and D). The percentage of antigen-specific IFN-γ^+ $CD4^+$ cells and IFN-γ^+ $CD8^+$ cells in NS-treated mice were (0.12 ± 0.02)% and (0.29 ± 0.02)%, and were little varied in mice immunized with HBsAg alone and HB vaccine groups. Co-administration of 1.0 mg of CS3 as adjuvant significantly increased the percentage of IFN-γ-producing CD4 and CD8 cells to (1.50 ± 0.59)% and (3.76 ± 0.59)%, respectively. The result of antigen-specific IL-4 producing $CD4^+$ cells was approximately in accordance with that of the cytokines detection in spleen supernatant (Fig. 4E). CS3 could inhibit HBsAg-induced IL-4 expression in a dose-independent manner. These results also indicated that CS3 and curdlan may act as a Th1-type immunomodulator in mice immunized with HBsAg.

Our previous study showed that CS3 and curdlan could increase the production of Th1- and Th17-polarizing cytokines, IL-12p70 and IL-6 in immature BMDCs (Li, Sheng, et al., 2013; Li, Zhang, et al., 2013), and other studies showed that dectin-1 agonist curdlan promoted Th1 and Th17 immunity (Leibundgut-Landmann et al., 2007), induced IL-10-producing $CD4^+$ T cells and inhibited OVA-induced Th2 cytokine production in the airways (Kawashima et al., 2012). Thus, this Th1 type cellular immune response of CS3 and curdlan could make up the defect of alum used an adjuvant.

3.3.6. *Effect of CS3 on HBsAg-specific antibody and immunoglobulin subtypes production in mice sera*

To estimate the effect of CS3 in enhancing the HBsAg-specific antibody response, serum antibody titers were measured 7 days after each immunization. As shown in Fig. 5, the specific-HBsAg IgG was not detectable (N.D.) in NS group. Mice immunized with HBsAg alone developed significant antibody response only after the third antigen inoculum, while HB vaccine after the secondary immunization. Conversely, mice receiving the antigen together with CS3 at the dosage of 1.0 mg already showed high and significant levels 7 days after the primary immunization, which were highly increased by the second and third challenges. CS3 at lower concentrations and curdlan also showed markedly high level of anti-HBsAg titers after the third immunization.

A clear difference between the adjuvant effect of CS3 and alum was evident when evaluating the titers of antigen specific IgG1 and IgG2a subclasses 7 days after the tertiary immunization. As shown in Table 3, HB vaccine increased the production of IgG1, but not IgG2a, whereas CS3 increased the titer of IgG2a but decreased that

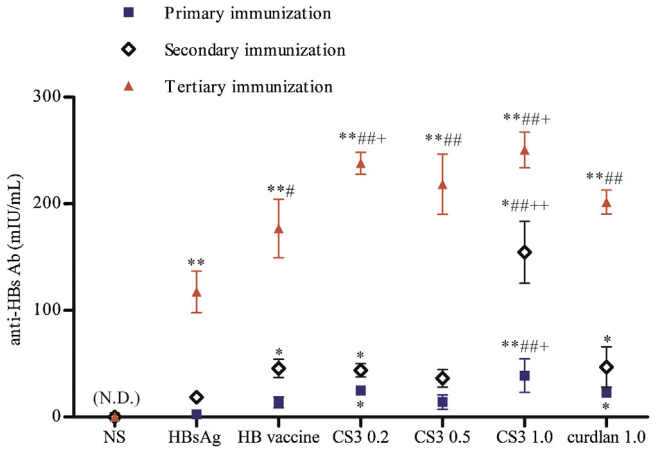

Fig. 5. Effect of CS3 on HBsAg-specific antibody production in mice sera. The sera were collected from six mice/group 7 days after each immunization. $^{*}p<0.05$, $^{**}p<0.01$, compared with NS group; $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared with HBsAg alone group; $^{+}p<0.05$, $^{++}p<0.01$, compared with HB vaccine group. Data represents mean ± SEM ($n=6$).

Table 3
Effect of curdlan sulfate on HBsAg-specific antibody subtypes production.

Group	IgG1	IgG2a	IgG2a/IgG1
NS	N.D.	N.D.	–
HBsAg alone	2.12 ± 0.28	1.81 ± 0.48	0.85 ± 0.12
HB vaccine	2.14 ± 0.22	1.47 ± 0.12	0.73 ± 0.09
HBsAg + CS3 0.2 mg/mice	1.31 ± 0.48$^{\#,+}$	2.41 ± 0.18^{++}	2.03 ± 0.80
HBsAg + CS3 0.5 mg/mice	1.18 ± 0.60$^{\#,+}$	2.48 ± 0.62$^{\#,++}$	2.83 ± 2.19
HBsAg + CS3 1.0 mg/mice	1.03 ± 0.47$^{\#\#,++}$	2.80 ± 0.10$^{\#\#,++}$	3.36 ± 2.15$^{\#,+}$
HBsAg + curdlan1.0 mg/mice	0.72 ± 0.02$^{\#\#,++}$	2.05 ± 0.30	2.82 ± 0.41

The sera from immunized mice were collected 7 days after the tertiary and evaluated by ELISA for anti-HBsAg specific IgG1 and IgG2a. The values are the absorbance of OD_{450}, and each value represents the mean ± SEM ($n = 6$). Data are the mean of six mice/group within one experiment representative of three performed. N.D. indicates that anti-HBsAg antibody at the sera of mice in under detection level.

$^{\#}$ Compared with HBsAg alone group, $p < 0.05$.
$^{\#\#}$ Compared with HBsAg alone group, $p < 0.01$.
$^{+}$ Compared with HB vaccine group, $p < 0.05$.
$^{++}$ Compared with HB vaccine group, $p < 0.01$.

of IgG1 dose-dependently. By calculating the ratio between IgG2a and IgG1 in the anti-HBsAg antibody response, immunization with CS3 at 1.0 mg was approximately 4-fold of that with HBsAg alone, and 4.6-fold of that with HB vaccine. Therefore, the anti-HBV antibody response to the CS3 adjuvant vaccine is strongly biased toward a Th1 type response, as compared to that of alum adjuvant that biases it toward Th2.

4. Conclusion

CS3 has been demonstrated to inhibit HBV infecting of HepG2 and HepaRG cells, and the mechanism may dependent on the sulfation groups on the polysaccharide chains. As an immunostimulatory agent, when administrated to mice immunized with HBsAg, CS3 could enhance the specific antibody response to HBsAg through amplification of multi-steps of immune response. A clear Th1 bias could be observed in CS3 and curdlan treated mice, higher IFN-γ and lower IL-4, as well as higher IgG2a/IgG1 ratio within the specific anti-HBsAg antibodies. Notably, although curdlan also exhibits Th1 type immune response to HBsAg, it can develop subcutaneous induration even after the first immunization (data not shown). This may due to its poor solubility in NS, which limits the application in some extent.

HBV infection is an important problem to human health worldwide, and the prevention and therapy are both issues of major importance. As the dectin-1 agonist, CS3 and curdlan can induce Th1 response and enhance cytotoxic T lymphocyte (CTL) function (Leibundgut-Landmann, Osorio, Brown, & Reis e Sousa, 2008), which can elicit cell-mediated immune responses to eliminate HBV completely. Since CS3 possesses not only the immunoadjuvant activity, but also anti-HBV infection potential, so it may constitute useful agents for vaccination and immunotherapy.

Acknowledgments

This work was supported by the National Basic Research Program of China (973 Program) (No. 2012CB822102) and Taishan Scholar Scheme of Shandong Province of China (Special Term Expert for Pharmacy) (No. 2012GSF30022).

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