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Preparation and *in vitro* immunomodulatory effect of curdlan sulfate

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ABSTRACT

Different curdlan sulfates were synthesized by modification of curdlan with sulfur trioxide–pyridine complex, and the one with the highest sulfur content (9.23 ± 0.16) %, CS3, was used to study the immunomodulating effect on murine RAW264.7 macrophages and bone marrow derived dendritic cells (BMDCs). The results showed that the treatment of macrophages with CS3 could not only increase the nitric oxide (NO) release and the cytokines TNF-α, IL-6 and IL-1β production significantly, but also enhance the inducible NOS (iNOS) expression, NF-κBp65 nuclear translocation, Erk1/2 and SAPK/JNK phosphorylation. The combination of CS3 with GM-CSF upregulated immature BMDCs to express major histocompatibility complex II (MHCII) and CD11c surface markers, CD40, CD80 and CD86 costimulatory molecules, as well as the cytokines of IL-12p70 and IL-6. The surface plasmon resonance (SPR) analysis found that CS3 exhibited binding affinity against dectin-1. These results suggested that curdlan sulfate can possibly be developed as a new immunotherapy agent and anti-viral vaccine adjuvant.

1. Introduction

Curdlan is an extracellular polysaccharide produced by the bacteria of *Alcaligenes faecalis* var. *myxogenes* 10C3, mutant K. It is the structurally simplest kind of 1,3-β-D-glucans with no glycosyl side chains, but has poor solubility in water, alcohols and most organic solvents (Harada, Masaki, & Saito, 1968; Mclntosh, Stone, & Stanisich, 2005). The poor solubility of curdlan may be due to its triple-stranded helical or single helix conformation in its powder state, and the formation of inter- or intra-molecular hydrogen bonds in water. This conformation will be transformed into random coil conformation with the hydrogen bonds broken when curdlan is dissolved in 0.19–0.24 M NaOH solution (Funami, Funami, Yaha, & Nakao, 2000). The molecular weight (MW) of natural curdlan is usually high, ranging from tens of thousands to millions Dalton (Da). So far, curdlan has been widely used in food industry as an additive, and it is the third bacteria extracellular polysaccharide approved by FDA in 1996. Curdlan can be used as a food additive because it is safe and its producing bacterium, *A. faecalis* var. *myxogenes*, is non-pathogenic and non-toxicogenic (Spicer, Goldenthal, & Ikeda, 1999). More importantly, curdlan has the ability of thermal-gelling, for example, its aqueous suspension will turn to gel once it is heated to 55 °C or above, which endows curdlan with thermostability, frost resistance and water-binding property (Mclntosh et al., 2005).

In addition to its applications in food industry, curdlan also has been discovered to have pharmacological activities, such as anti-tumor (Sasaki, Abiko, Sugino, & Nitta, 1978), anti-oxidative (Kishk & AI-Sayed, 2007), and immunomodulating activities (Hida et al., 2009). Many methods have been developed to improve the solubility of curdlan to expand its applications, endowing curdlan derivatives with anti-tumor, anti-HIV, anti-coagulation, improvement of wound healing activities as well (Berdal et al., 2007; Demleitner, Kraus, & Franz, 1992; Jin, Zhang, Yin, & Nishinari, 2006; Qian, Wu, Pan, & Xia, 2012; Yamamoto et al., 1990). The sulfation modification of curdlan is one convenient and feasible method to improve its solubility, with approved anti-coagulant and anti-HIV activities (Alban, Jeske, Welzel, Franz, & Fareed, 1995; Yamamoto et al., 1990; Yoshida et al., 1995). Curdlan sulfate has been studied as an anti-HIV agent since 1990 (Kaneko et al., 1990), and the study has carried out in clinical phase II with good efficiency and low toxicity (Gordon et al., 1997). What is more, dectin-1, a β-glucan receptor mainly expressed on dendritic cells, monocytes/macrophages and neutrophils (Taylor et al., 2002), has been found to be the receptor of curdlan mediating the

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immunomodulating activity (Ferwerda, Meyer-Wentrup, Kullberg, Netea, & Adema, 2008). Therefore, we consider that curdlan sulfate may also possess good immunomodulating activities and act as a good anti-viral agent or vaccine adjuvant.

In this study, we had prepared curdlan sulfates with different MWs and sulfur contents by the chemical modification with sulfur trioxide–pyridine complex and characterized the derivatives. The immunomodulating activity of curdlan sulfate was studied, including its effect on murine RAW264.7 macrophage cell line and the BMDCs. The binding affinity of curdlan sulfate to recombinant dectin-1 was also studied by SPR technology.

2. Materials and methods

2.1. Reagents and antibodies

Curdlan was obtained from Zhongke Biotechonlogy Co. (Shandong, China) and Sigma–Aldrich Chemical Co. (St. Louis, Missouri, USA). Sulfur trioxide–pyridine complex was purchased from Hongda Chemical Co. (Shandong, China). Lipopolysaccharides (LPS) and FITC-dextran were purchased from Sigma–Aldrich Chemical Co. Recombinant mouse dectin-1/CLEC7A, and recombinant mouse TLR2 Fc Chimera were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant murine GM-CSF and IL-4 were from PeproTech (Rocky Hill, NJ, USA). ELISA Kits for IL-1β, IL-6, TNF-α, and IL-12p70 determinations were from ExCell Biology, Inc. (Shanghai, China). Anti-mouse FITC-CD11c, PE-MHCII (I-A/I-E), FITC-CD80 and PE-CD86 were all purchased from eBioscience (San Diego, CA, USA). Rabbit polyclonal anti-iNOS, rabbit monoclonal anti-phospho-Erk1/2, rabbit monoclonal anti-phospho-SAPK/JNK, and rabbit monoclonal anti-NF-κBp65 antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Rabbit polyclonal anti-phospho-p38 was purchased from ImmunoWay Biotechnology Co. (Newark, DE, USA). Rabbit polyclonal anti-GAPDH antibody was from Goodhere Biotechnology Co., Ltd. (Hangzhou, China). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All other chemicals and reagents used were analytical grade.

2.2. Preparation of curdlan sulfate

Sulfation of curdlan was prepared according to the method of Yamamoto et al. (1996) with some modifications. Briefly, 1 g of curdlan was dissolved in 100 mL of DMSO (previously dried with molecular sieve 4A) by stirring overnight at room temperature. Then 2.5 g of sulfur trioxide–pyridine complex was added to the reaction system and heated up to 85 °C. This reaction was lasting for 1 h. After that, the reaction mixture was cooled with cold water and 500 mL of methanol with 5% sodium acetate was added. After 3–4 h placement, the product was precipitated out, centrifuged and dissolved in water. The resulting solution was desalted and concentrated through an ultrafiltration membrane with a MW cut-off of 10,000 (Millipore), and then dried in a vacuum freezing dryer and the compound named as CS1 was got. In the same manner, we changed the reaction temperature to 75 °C and 65 °C and got another two compounds named as CS2 and CS3, respectively. Meanwhile, the sulfated compound, CSIII, was prepared by curdlan from Sigma–Aldrich with the same reaction condition as CS1.

2.3. Characterization of curdlan sulfate

The structures of curdlan sulfates were confirmed by FTIR spectroscopy, which were taken with KBr pellets on an FT-IR Nicolet 6700 spectrophotometer in the range of 4000–400 cm^{-1} with a revolution of 4.00 cm^{-1}. Curdlan from Sigma–Aldrich and CSIII was used as the reference substances. The MWs of CS1, CS2, CS3 and CSIII were determined by SEC-MALLS (size-exclusion chromatographic analysis with multi-angle light scattering) system consisting of an Agilent 1100 HPLC with RI detector and Wyatt DAWN EOS detector (Wyatt Technology Corp., USA) with a flow rate of 0.5 mL/min. Samples were prepared to a 0.5 mg/mL solution and a refractive index increment (dn/dc) of 0.135 was used in the processing of the data. The sulfur contents of curdlan sulfates were analyzed by element analysis using Elementar Vario EL III Analyzer (Elementar Analysensystem GmbH, Germany).

2.4. Effects of curdlan sulfate on RAW264.7 macrophages

2.4.1. RAW264.7 culture

RAW264.7, a mouse macrophage cell line, was obtained from the Shanghai Cell Bank, the Institute of Cell Biology, China Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco), penicillin-streptomycin (100 IU/mL to 100 μg/mL), 2 mM glutamine, and 10 mM HEPES in a humid atmosphere (5% CO_2, 95% air) at 37 °C, fed every 2–3 days and harvested by brief treatment with 0.02% EDTA-0.25% trypsin.

2.4.2. Cell proliferation assay

RAW264.7 cell proliferation was measured by conventional MTT assay. The cells were cultured in 96-well plates at a density of 3×10^3 cells/well in 100 μL medium with different final concentrations of curdlan sulfate (1, 10, 100 μg/mL, dissolved in serum-free medium) for 24 or 48 h at 37 °C and 5% CO_2. 20 μL of 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 at 570 nm was measured by a microplate reader (Bio-Rad). Each experimental condition was tested in triplicate.

2.4.3. NO analysis

Nitrite accumulation in the medium was used as an indicator of NO production as previously described (Green et al., 1982), and colorimetric assays with Griess reagent were used to detect NO levels (Mazzone, Rigato, & Tiribelli, 2010). RAW264.7 cells were seeded in a 24-well plate at a density of 2×10^6 cells/mL, 1 mL of each well. After the cells were cultured with curdlan sulfate (1, 10, 100 μg/mL), curdlan (100 μg/mL, curdlan was solubilized with 0.15 M NaOH, and diluted 100-fold with the medium) or LPS (1 μg/mL, dissolved in serum-free medium) for 24 h, the supernatant were collected and the NO level in the medium was determined by the Nitric Oxide Assay Kit (Beyotime, Jiangsu, China) based on the Griess reagent method.

2.4.4. FITC-dextran internalization experiment

After the cells were incubated with curdlan sulfate (1, 10, 100 μg/mL), curdlan (100 μg/mL) or LPS (1 μg/mL) for 24 h, 1 mg/mL FITC-dextran (Sigma) was added to each well and incubated at 37 °C for 30 min. The cells cultured with FITC-dextran at 4 °C for 30 min were used as a blank control. The reaction was stopped by cold PBS containing 2% fetal serum. The cells were washed three times and the internalization of FITC-dextran to the cells was analyzed by flow cytometer (FACS calibur, Becton Dickinson).

2.4.5. Cytokines assays

RAW264.7 cells were seeded in a 96-well plate at a density of 2×10^6 cells/mL, 100 μL each well. After being incubated with curdlan sulfate (1, 10, 100 μg/mL), curdlan (100 μg/mL) or LPS (1 μg/mL)

for 6 h (TNF-α) or 24 h (IL-6 and IL-1β), the supernatant were collected and the cytokines level of TNF-α, IL-6 and IL-1β were determined by ELISA kit according to the manufacturer's instructions.

2.4.6. Western blot analysis

RAW264.7 cells (4×10^6 cells) seeded in 6-well plates were treated with different concentrations of curdlan sulfate for 24 h, and then the medium was removed and the cells were washed with PBS, lysed in 500 μL of lysis buffer and shaken at 4 °C for 20 min. Total protein was determined using the BCA (bicinchoninic acid) method. Aliquots of 35–40 μg protein in the cell extracts were mixed with 5 × SDS loading buffer, denatured in boiling water for 5 min, loaded and separated by 10% SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF) membrane. After the non-specific binding was blocked with TBST (20 mM Tris-buffered saline and 0.1% Tween) containing 5% nonfat dry milk for 1–2 h at room temperature, the membranes were incubated with polyclonal anti-iNOS, monoclonal anti-phospho-Erk1/2, monoclonal anti-phospho-SAPK/JNK or polyclonal anti-phospho-p38 antibodies under shaking at 4 °C overnight, followed by incubation with HRP conjugated secondary antibody. Then the proteins were detected using enhanced chemiluminescence kit (ECL, Millipore) according to manufacturer's instructions (Qi et al., 2008). For the detection of nuclear NF-κBp65 protein, the nuclear proteins were prepared using a commercial extraction kit (BestBio, Shanghai, China) and then the Western bolt analysis was performed as mentioned above. GAPDH was used as the loading control for the proteins.

2.5. Effects of curdlan sulfate on the BMDCs

2.5.1. Generation and culture of mouse BMDCs

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. Male BALB/c mice (7 weeks old) were obtained from the Laboratory Animal Center, Shandong University (Jinan, China). Preparation of mouse bone marrow derived dendritic cells (BMDCs) was performed according to previous description (Inaba et al., 1992). Briefly, the mice were sacrificed by cervical dislocation and put into 70% alcohol for 10 min. The femurs of the mice were separated, and the muscle tissues were removed with gauze. The cleaned femurs were placed in 70% alcohol for 1 min, washed twice with PBS, and then transferred into a fresh dish with 10 mL of RPMI 1640 medium supplemented with 10% FBS. The bone ends were cut with scissors, and the bone marrow cells were flushed out using 2 mL of PBS with a syringe. Then, the bone marrow cells were passed through a nylon mesh to remove small pieces of bone and debris, and the red blood cells were lysed by ammonium chloride. 1×10^7 cells in 1 mL medium were placed in each well of 6-well plates supplemented with 100 ng/mL GM-CSF and 50 ng/mL IL-4. The cultures were fed every 2 d, and nonadherent cells were gently washed away. At day 6, the loosely attached DCs were collected.

2.5.2. Detection of DC maturation markers

The collected DCs were incubated with curdlan sulfate (1, 10, 100 μg/mL), curdlan (100 μg/mL) or LPS (1 μg/mL) in GM-CSF-containing cultures for 48 h in 6-well plates at 37 °C, and then the cells were stained with FITC-CD11c, PE-MHCII (I-A/I-E), FITC-CD80, and PE-CD86, and analyzed by flow cytometry.

2.5.3. IL-12 and IL-6 assays

The collected DCs were treated by curdlan sulfate, curdlan or LPS combined with GM-CSF for 48 h in a 96-well plate at 37 °C, and IL-12p70 and IL-6 in the supernatant were detected by ELISA kit according to the manufacturer's instructions.

2.6. Affinity assay of curdlan sulfate to dectin-1

The affinity of curdlan sulfate to recombinant mouse dectin-1 was examined by SPR analysis performed using BIAcore 3000 (GE Healthcare) according to the manufacturer's instruction. Before the analysis, recombinant mouse Dectin-1/CLEC7A (R&D Systems) was immobilized on sensor chip CM5 using amine coupling method. The resultant sensor chip was stored in the running buffer (HBSEP: 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 3 mM EDTA and 0.005% of surfactant P20), and the temperature of the sensor chip was maintained at 25 °C for all experiments. Curdlan sulfate dissolved in running buffer at a series of concentrations was injected into the channels to record the signals with BIAcore program (GE). After each analysis, the sensor chip was regenerated by 10 mM glycine–HCl (pH 2.5). Affinity constants were calculated using BIA evaluation 4.1 software by globally fitting the association and dissociation phases of overlay plots to a 1:1 Langmuir binding model.

2.7. Statistical analysis

Data are expressed as mean ± SD, 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. Curdlan sulfate

Through controlling the reaction temperature, four kinds of curdlan sulfates with different sulfur contents and MWs were obtained (Table 1). The extent of sulfation of all samples was relatively low compared to previous work, which showed the S% was more than 10% (Yamamoto et al., 1996), as deduced from the element analysis results. This may be caused by the exchange of piperidine sulfate to sulfur trioxide–pyridine complex, as well as the curdlan we used had high MW. Curdlan sulfate has been reported to be synthesized by three conventional chemical methods (Yoshida et al., 1995). Since DMSO is much safer and less toxic than pyridine as a solvent, and the preparation of sulfating reagent piperidine-*N*-sulfonic is complicated (Nagasawa & Yoshidome,

Table 1
The prepared cudlan sulfates with different molecular weight (MW), sulfur (S) content and the binding affinity K_D (equilibrium dissociation constant) values.

Sample	CS1	CS2	CS3	CSIII
MW (kDa)	25.87	87.03	171.9	62.54
S content (%)	4.03 ± 0.3	6.27 ± 0.03	9.23 ± 0.16	7.59 ± 0.11
K_D (M)	$(8.16 \pm 4.60) \times 10^{-9}$	$(3.80 \pm 1.23) \times 10^{-6}$	$(4.24 \pm 2.47) \times 10^{-5}$	$(2.95 \pm 4.69) \times 10^{-6}$

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

1969), we choose DMSO/sulfur trioxide–pyridine as the reaction system. Moreover, higher sulfur content of curdlan sulfate may lead to an increase in activated partial thromboplastin time and the prolongation of the coagulation time, which would to be side effects of curdlan sulfate for clinical use (Havlik et al., 2005). Besides, the sulfur content and MW of curdlan sulfate were closely related to the reaction temperature, the lower the reaction temperature, the higher sulfur content and MW of curdlan sulfate. This could be an indicator for the preparation of different kinds of curdlan sulfate derivatives. Fig. 1 shows the FT-IR spectra of different curdlan sulfates. As shown in Fig. 1, the presence of the peaks at 1259 cm^{-1}, 1258 cm^{-1}, 1258 cm^{-1} and 1262 cm^{-1} for CSIII, CS1, CS2 and CS3, respectively, represent the asymmetrical S=O stretching vibration. The other characteristic absorption bands at 816 cm^{-1}, 812 cm^{-1}, 814 cm^{-1}, and 818 cm^{-1} for CSIII, CS1, CS2 and CS3 are ascribed to the symmetrical C—O—S stretching vibration (Yang, Du, Wen, Li, & Hu, 2003). These spectra confirmed that the sulfate groups had been incorporated into the curdlan backbone. We chose CS3 as the study object in the following activity studies as it had the highest sulfur content among the prepared compounds, and we considered the sulfation modifications may improve the activity of curdlan.

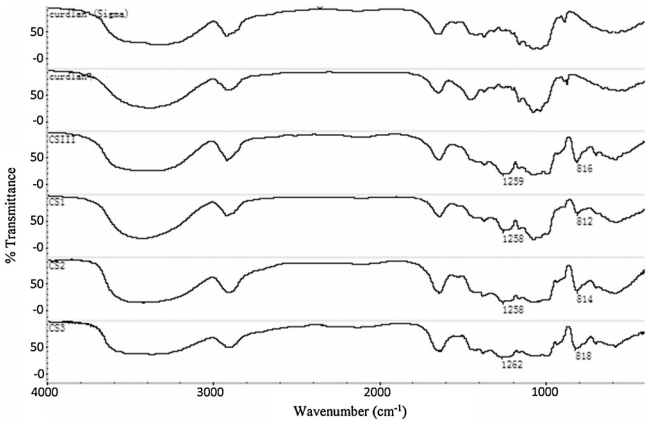

Fig. 1. FT-IR spectra of curdlan and its sulfate derivatives. From top to bottom, they were curdlan form Sigma–Aldrich, curdlan from Zhongke, CSIII, CS1, CS2, CS3, respectively.

3.2. Effect of curdlan sulfate on RAW264.7 macrophages

Curdlan has been proved to activate macrophages by inducing the production of iNOS, TNF-a, MIP-2 (macrophage inflammation protein-2) and the activation of NF-κB through a MyD88 (myeloid differentiation primary response gene 88)-dependent signaling pathway in earlier studies (Hoebel et al., 2003). When the macrophages are activated, they will produce NO, which is crucial in killing microbes and tumor cells. After uptaking a pathogen, the macrophage cell will present the antigen of the pathogen to the corresponding helper T cell through high expression of costimulatory molecules, such as CD80 and CD86, acting as an important antigen-presenting cell (APC) regulating innate and adaptive immune

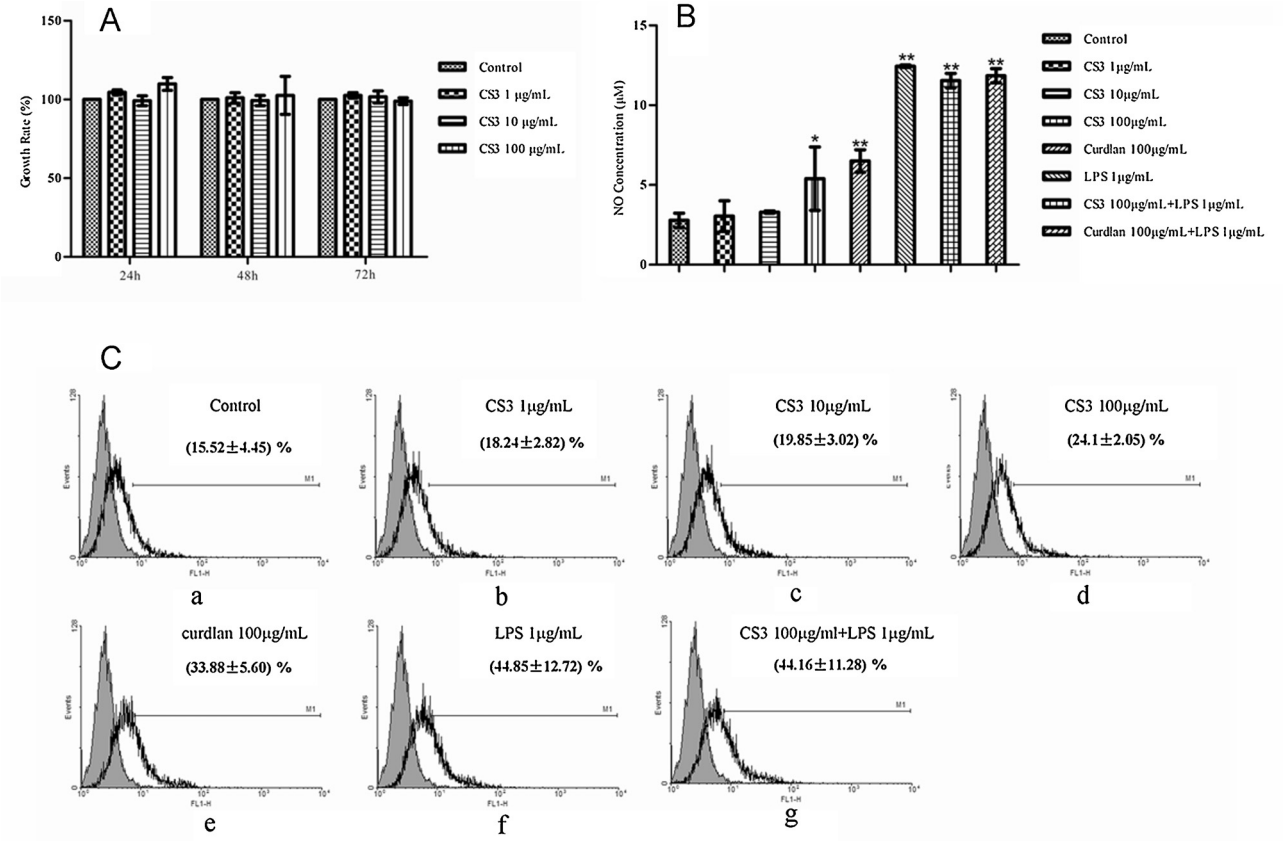

Fig. 2. Effects of CS3 and curdlan on RAW264.7 cells. (A) Effect of CS3 and curdlan on proliferation of RAW264.7 cells after the treatment with CS3 for 24, 48, and 72 h. (B) Effect of CS3 and curdlan on NO release of RAW264.7 cells. Cells were pretreated with CS3 (1, 10, or 100 μg/mL), curdlan (100 μg/mL), LPS (1 μg/mL), or the combination of LPS with CS3 or curdlan for 48 h. The nitrite levels in the supernatant were determined using Griess reagent. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with control group ($n = 3$). (C) Effect of CS3 and curdlan on the phagocytosis of RAW264.7 cells. (a) Control; (b) CS3 1 μg/mL; (c) CS3 10 μg/mL; (d) CS3 100 μg/mL; (e) curdlan 100 μg/mL; (f) LPS 1 μg/mL; (g) CS3 100 μg/mL + LPS 1 μg/mL. The figure shown was one representative FACS plots of three independent experiments, and the data marked on the figure represented the percentage of positive cells of three experiments (mean ± SD). Treatment with CS3 was not significant, $p > 0.05$, whereas treatment with curdlan, LPS, as well as the combination groups was significant, $p < 0.01$, compared with control.

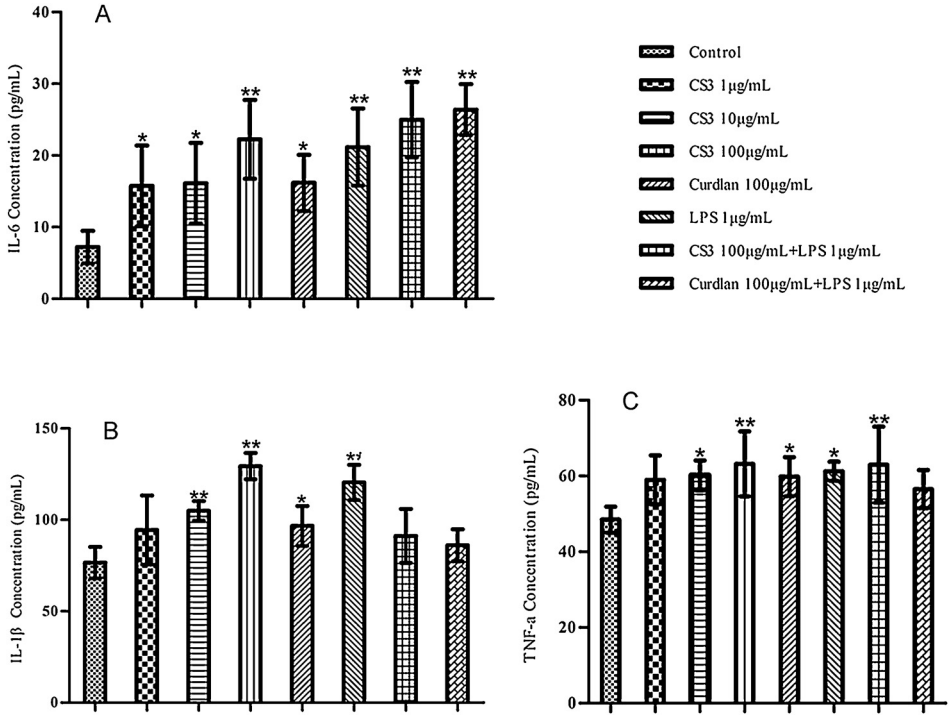

Fig. 3. Cytokines production of RAW264.7 cells after the treatment with CS3 and curdlan. IL-6 and IL-1β (24-h stimulation) and TNF-a (6-h stimulation) were measured with ELISA kits. The data represented three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with control.

responses during microbial infections (Kataoka, Muta, Yamazaki, & Takeshige, 2002; Lehtonen et al., 2007). Therefore, we detected the effects of curdlan sulfate on RAW264.7 cells.

3.2.1. Effect on RAW264.7 proliferation

It was necessary to evaluate whether curdlan sulfate has cytotoxic effect or proliferation effect on RAW264.7 cells before further tests. MTT assay indicated that CS3 did not show cytotoxic or stimulation effect on RAW264.7 cells in 24, 48 or 72 h, under the tested concentrations (Fig. 2A). Based on this, the measurement of phagocytosis, NO and cytokines production can well represent the cell function without any changes in cell quantity.

3.2.2. Effect on NO release

NO is an important mediator produced by macrophages inducible nitric oxide synthase, and contributes to diverse biological processes in immune systems, such as inhibiting the growth of bacteria, fungi and parasites (Lowenstein, Dinerman, & Snyder, 1994). The effects of curdlan sulfate and curdlan on the NO production of RAW264.7 were determined by Griess assay. The nitrate concentrations of the culture supernatants were significantly increased by CS3 ($p < 0.05$) or curdlan ($p < 0.01$) at the concentration of 100 μg/mL (Fig. 2B). Also, the combination of curdlan sulfate or curdlan with LPS had no synergistic effect in NO release.

3.2.3. Effect on FITC-dextran internalization into RAW264.7 cells

The FITC-dextran internalization into RAW264.7 cells was analyzed using flow cytometry. As shown in Fig. 2C, at the tested concentrations, CS3 seemed to increase the phagocytosis of the cells in a dose-dependent manner, however, this effect was not significant even at 100 μg/mL compared with the control ((24.1 ± 2.05)% *vs.* (15.52 ± 4.45)%, $p > 0.05$, $n = 3$). Curdlan remarkably stimulated phagocytosic ability of RAW264.7 ((33.88 ± 5.60)%, $p < 0.01$ *vs.* control, $n = 3$) as LPS action ((44.85 ± 12.72)%, $p < 0.01$ *vs.* control, $n = 3$). And the addition of CS3 to LPS did not increase the effect of LPS acting alone ((44.16 ± 11.28)%, $n = 3$).

3.2.4. Effect on IL-6, IL-1β, and TNF-α production

Activated macrophages are a major source of many cytokines involved in immune system response to tissue injury, as well as regulating the actions of many cell types important for the host response to infection and inflammation, including IL-1, IL-6, IL-10, TNF-a, IFN-γ, and so on (Cavaillon, 1994). We tested the effects of CS3 on the production of IL-6, IL-1β, and TNF-a of RAW264.7, and found that CS3 could enhance the cytokines release significantly, similar to curdlan. As shown in Fig. 3A, compared with control, CS3 can enhance IL-6 concentration significantly at the tested concentrations of 1, 10 μg/mL ($p < 0.05$), and 100 μg/mL ($p < 0.01$), as well as curdlan at 100 μg/mL ($p < 0.05$). When CS3 was administered combined with LPS, the enhancement effect showed no significant difference with each of them used alone. While, the combination of curdlan and LPS showed an improved effect compared with curdlan used alone, which may be caused by the effect of LPS. The IL-1β and TNF-α concentrations of the culture supernatant were significantly increased by CS3 (10 and 100 μg/mL, $p < 0.01$) and curdlan (100 μg/mL, $p < 0.05$) in a dose-dependent manner (Fig. 3B and C), and CS3 (100 μg/mL) showed more potent ability than curdlan in IL-1β production ($p < 0.01$). Similarly, the combination usage of CS3 or curdlan with LPS did not show any synergistic effect, and even attenuated their ability in some extent. These results suggest both CS3 and curdlan can stimulate IL-6, IL-1β, and TNF-α production of RAW264.7, however, they do not show synergistic effect with LPS.

3.2.5. Effect on iNOS, phospho-Erk1/2, phospho-SAPK/JNK, phospho-p38, and NF-κBp65 expression

The iNOS is one kind of nitric oxide synthases catalyzing the production of NO from L-arginine, which is not expressed constitutively but can be induced in response to specific signals in cells

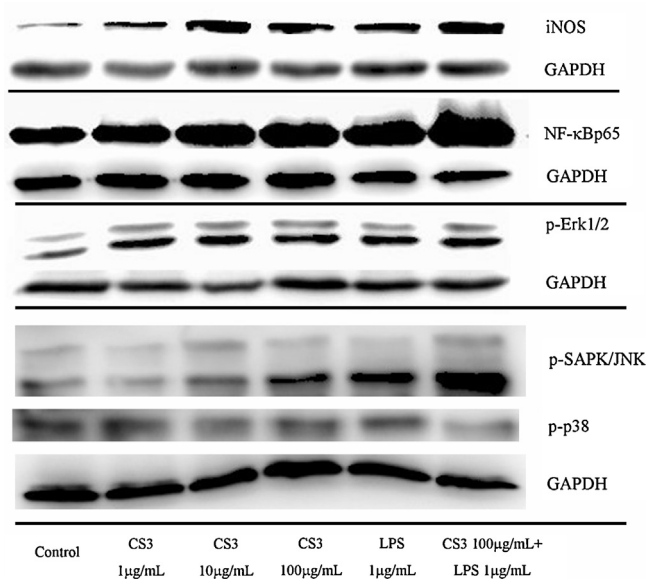

Fig. 4. Effect of CS3 on iNOS, NF-κBp65, phospho-Erk1/2, phospho-SAPK/JNK, and phospho-p38 expression of RAW264.7 cells. Data were representative of three independent experiments.

such as hepatocytes, fibroblasts, and macrophages (Nathan & Xie, 1994), and iNOS derived NO plays important roles in anti-microbial and anti-tumor defenses in mammals (Mayer & Hemmens, 1997). Mitogen-activated protein kinase (MAPK) cascade signaling pathway is one of the most important signaling pathways that governs the cells growth, proliferation, differentiation and survival and participate in cancer, immunological, and inflammatory diseases. Mammalian MAPK pathway contains four main signaling modules, Erk1/2, SAPK/JNK, p38, and Erk5 (Orton et al., 2005). Once being activated, MAPK signaling can translocate from the cytoplasm to the nucleus and regulate gene transcription through affecting chromatin structure and modifying the activity of transcription factors (Murphy, MacKeigan, & Blenis, 2004; Orton et al., 2005). NF-κB, one of these crucial transcription factors, playing a pivotal role in regulating the immune responses, is also involved in iNOS transcription (Baeuerle & Henkel, 1994; Bogdan, 2001).

To determine whether CS3 interfered with MAPK pathway and influenced NF-κB and iNOS expression, we analyzed the cytoplasmic and nuclear cell lysates by Western blot with the specific phospho-antibodies against Erk1/2, SAPK/JNK, and p38 MAP kinases, as well as NF-κB p65 and iNOS antibodies. The results (Fig. 4) showed that at the tested concentration, CS3 could stimulate phosphorylation and elevate the expression of Erk1/2 and SAPK/JNK, but no apparent difference in the phosphorylation of p38 was observed between the different stimulated groups, meaning CS3 can activate MAPK pathway through Erk1/2, SAPK/JNK signalings. The expression of p65, a subunit of NF-κB, was also increased in this test, which represents the activation of NF-κB. In accordance with the results of NO assay (Fig. 2B), the substantial iNOS expression was induced by CS3 and the combination of CS3 with LPS notably. These results indicate that CS3 can activate MAPK signaling pathway, and further induce the translocation of NF-κB into nuclear, where it regulates the iNOS gene expression.

3.3. Effect of curdlan sulfate on the maturation of BMDCs

The state of maturation and cytokines production of DCs affect the differentiation of Th subset, such as Th1 and Th17, which play critical roles in immunity against foreign invaders. The maturation process of DCs involves the upregulation of MHC and costimulatory molecules and secretion of several proinflammation cytokines. Studies have proved the combination of curdlan and GM-CSF acted in synergy in stimulating immature BMDCs to potent effector DCs, by the performances of upregulating the expressions of a variety of cytokines, costimulatory molecules, chemokines, and receptor molecules (Min et al., 2012). However, stimulation of spleen DCs by curdlan alone displayed negative result on the maturation and cytokines production (Soltani, Kalantari, Karimi, Erfani, & Sarvestani, 2012). We want to find out whether the sulfation modification of curdlan will still maintain this activity.

3.3.1. Effect on the expression of BMDCs surface markers and costimulatory molecules

After 7 days of culture, the nonadherent cells were collected and treated with GM-CSF combined with CS3, curdlan or LPS for additional 48 h, and upregulated expression of MHCII, CD40, CD11c, as well as CD80 and CD86, were observed as shown in Figs. 5 and 6A. Compared with negative control (RPMI 1640), both CS3 and curdlan could markedly increase the expression of MHCII, CD40, and CD11c as assessed by the mean fluorescence intensity (MF) of the entire population of cells analyzed. The upregulation of MHCII, CD40, and CD11c expression by CS3 was dose dependent from 1 μg/mL to 100 μg/mL (Fig. 5A and B, $n = 3$). Likewise, the other maturation markers, CD80 and CD86 were also upregulated in response to CS3 and curdlan significantly (Fig. 6A) (CS3 1 μg/mL, $p < 0.05$; CS3 10, 100 μg/mL, curdlan 100 μg/mL, $p < 0.01$). The percentage of $CD80^+CD86^+$ cells stimulated by RPMI 1640, CS3 1 μg/mL, 10 μg/mL, 100 μg/mL, curdlan 100 μg/mL and LPS 1 μg/mL were $(19.16 \pm 2.94)\%$, $(30.60 \pm 2.77)\%$, $(40.0 \pm 3.83)\%$, $(44.31 \pm 2.78)\%$, $(47.73 \pm 4.14)\%$, $(45.48 \pm 4.14)\%$, respectively ($n = 3$). The data of Figs. 5 and 6A indicated CS3 and curdlan enhanced the phenotypic maturation of BMDCs.

3.3.2. Effect on the production of IL-12p70 and IL-6

The production of Th1- and Th17-deviating cytokines, IL-12p70 and IL-6, were also tested after the immature BMDCs were exposed to CS3 and curdlan in GM-CSF supplemented medium for 48 h. As shown in Fig. 6B, the cultured BMDCs became matured not only with the higher expression of key surface markers, but also the higher level generations of IL-12p70 and IL-6.

We also tested CS3 acted on immature BMDCs alone without GM-CSF and the results did not show a significant difference in the levels of surface markers expression and cytokines production (data not shown). These activated DCs can prime and induce cytotoxic T-lymphocyte (CTL) responses (Leibundgut-Landmann, Osorio, Brown, & Reis e Sousa, 2008), and change Treg cells into Th17 cells (Osorio et al., 2008).

3.4. Affinity of curdlan sulfate to recombinant dectin-1

Dectin-1 has been extensively identified as the primary pattern recognition receptor for β-glucan of fungal and bacterial cell walls, which is primarily expressed by cells of myeloid origin, including macrophages, neutrophils and DCs in mice (Brown & Gordon, 2003; Herre, Gordon, & Brown, 2004). In previous studies, it has been reported that curdlan can activate macrophages and immature BMDCs selectively *via* dectin-1/Syk pathway (Herre et al., 2004; Masuda, Togo, Mizuno, Konishi, & Nanba, 2012; Min et al., 2012; Osorio et al., 2008), and curdlan has been identified as the ligand of dectin-1 and used widely as the agonist of dectin-1 for research in immune system (Ferwerda et al., 2008; Gringhuis et al., 2009; Osorio et al., 2008).

Here we applied SPR technology with a BIAcore 3000 biosensor system to analyze the affinity of curdlan sulfate derivatives to recombinant mouse dectin-1. Dectin-1 was immobilized on the CM5 chip with a coupling capacity of approximately 5000 RU as

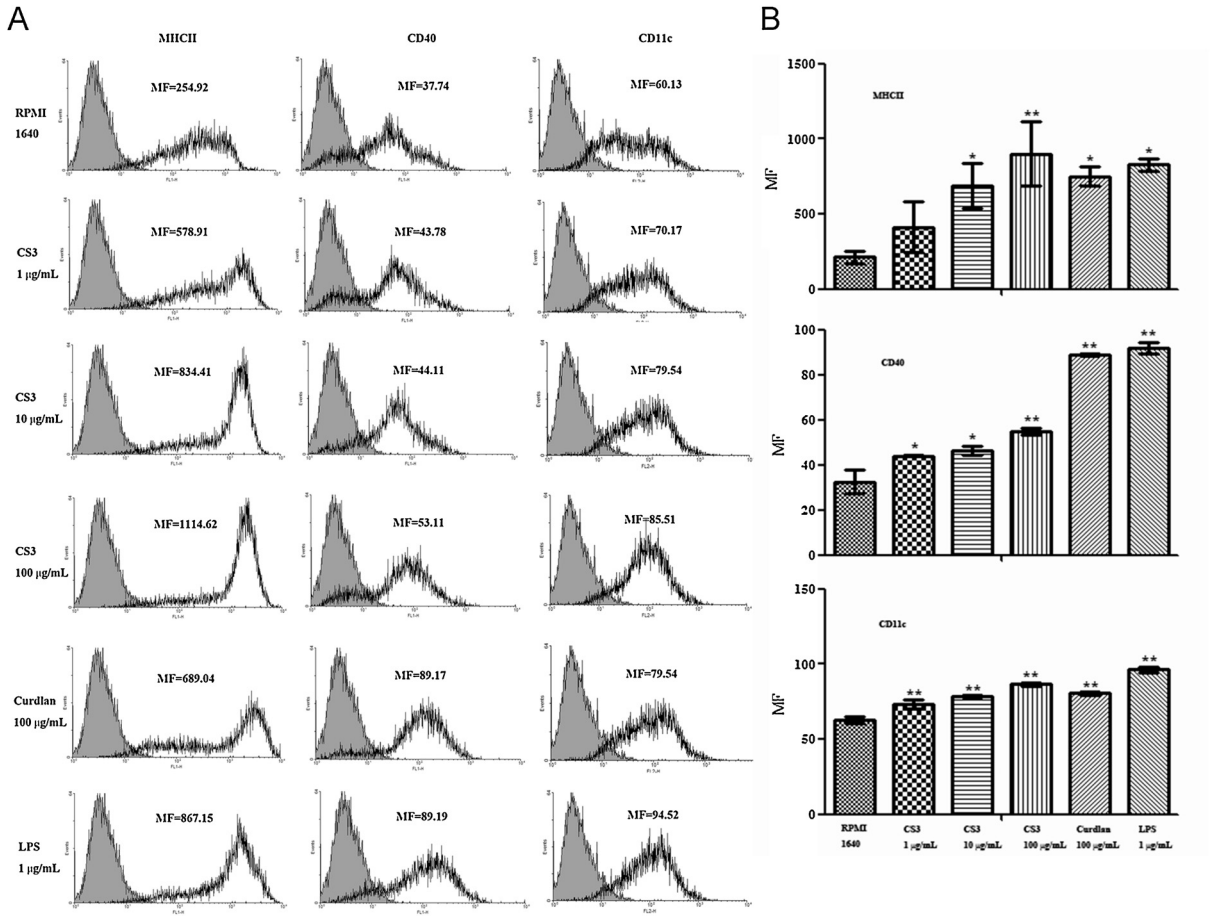

Fig. 5. Effects of CS3 treatment on the surface expression of MHCII, CD40 and CD11c of BMDCs. (A) Immature BMDCs were incubated with CS3, curdlan or LPS in the presence of GM-CSF for 48 h. Nonadherent cells were collected and analyzed for expression of MHCII, CD40 or CD11c by FACS. The data shown were representative FACS plots with mean fluorescence intensity (MF) values, $n = 3$. (B) The MF values of MHCII, CD40 and CD11c were calculated by three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with RPMI 1640 group.

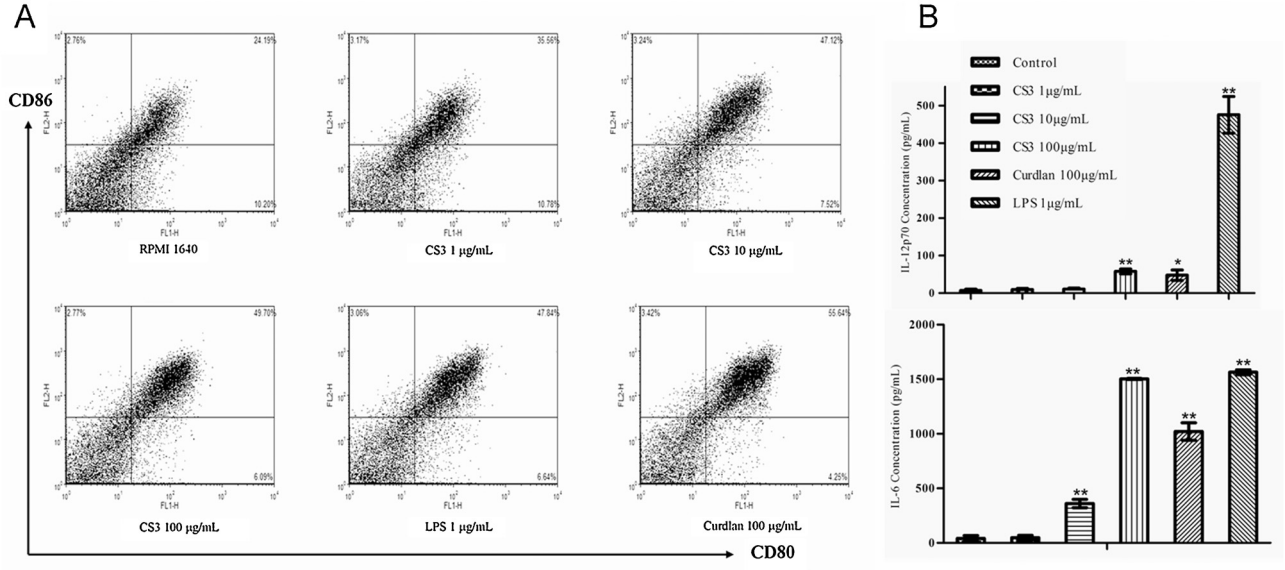

Fig. 6. Effects of CS3 on the expression of CD80, CD86, IL-12p70 and IL-6 of BMDCs. (A) Effect of CS3 on the surface expression of CD80 and CD86 of BMDCs. The percentage of $CD80^{+}CD86^{+}$ BMDCs was determined by flow cytometry after stained with FITC-CD80 and CD86 mAb. The data shown was one representative of three different experiments. (B) Effect of CS3 on the cytokines IL-12p70 (upper) and IL-6 (lower) secretion from cultured supernatant of BMDCs. Results were expressed as the mean ± SD of triplicate samples. CS3 (10, 100 μg/mL) and curdlan (100 μg/mL) led to an increase production of IL-12p70 and TNF-a, $^{*}p < 0.05$ or $^{**}p < 0.01$, compared with those in RPMI 1640.

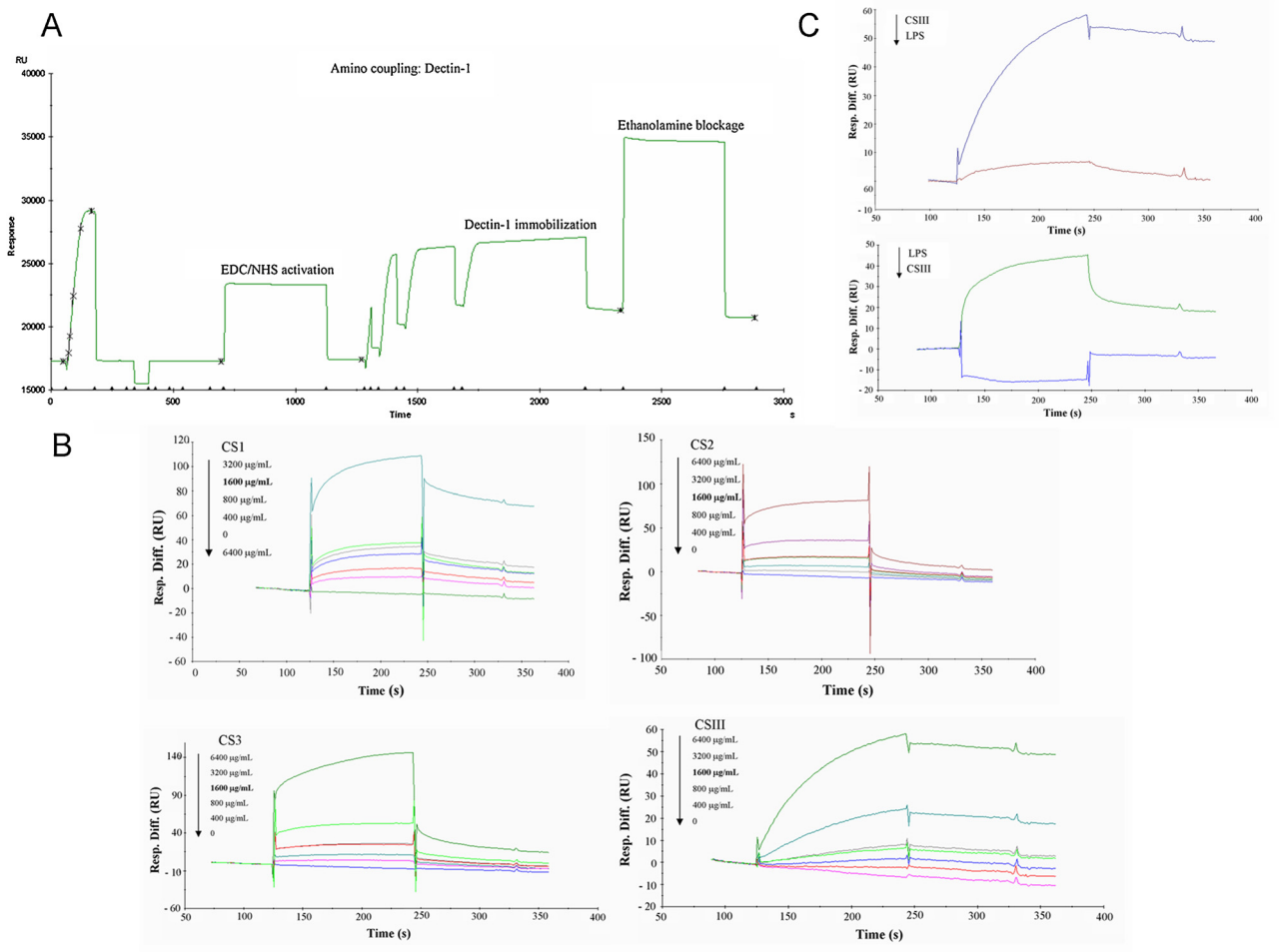

Fig. 7. Binding affinities of different curdlan sulfates against dectin-1 were evaluated by SPR. Six serial dilution concentrations of the four different curdlan sulfates were injected at 30 μL/min for 60 s association periods. The concentrations were shown next to the arrows. Bold figures indicated the repeated concentrations. The sensorgrams were obtained and the K_D values were analyzed using a global fit algorithm (BIA evaluation 3.1). (A) Immobilization of dectin-1 on CM5 sensor chips. (B) Kinetic-binding responses of CS1, CS2, CS3, and CSIII to dectin-1. (C) The sensorgrams of CSIII (800 μg/mL) and LPS (100 μg/mL) binding with dectin-1 or TLR2, respectively. CSIII showed high affinity with dectin-1 (upper), while LPS exhibited low affinity; LPS could bind with TLR2 whereas CSIII could not (lower).

shown in Fig. 7A. 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. The sensorgrams and K_D values are listed in Fig. 7B and Table 1. All of the curdlan sulfate derivatives prepared showed binding affinity toward dectin-1, and the K_D value had a tendency to decrease with the increases of MW and sulfur content, ranging from nanomole to micronmole grade, which may be caused by the increasing steric hindrance. Strangely, CS1 showed low affinity at the high concentration of 6400 μg/mL than other tested concentrations.

Previous study has reported that dectin-1 differentially interacted with 1,3-β-D-glucans over a wide range of binding affinities (2.6 mM-2.2 pM), and found that the binding affinity increased with the increase degree of polymerization and the presence of (1 → 6)-β-linked glucose side-chain branches. The 1,3-β backbone composed of seven or more glucose repeat subunits was assumed a helical conformation, which facilitates the interaction with the Trp221/His223 groove in dectin-1 (Adams et al., 2008). Curdlan had been reported to exhibit a single helix in water, and the single helix of these molecules associated to form local junction zones composed of double- or triple-stranded helices through hydrophobic or hydrogen bonds (Funami et al., 2000; Kataoka et al., 2002), and this may be the structure basis to bind with dectin-1. However, in our assay, the binding affinity of curdlan sulfate with dectin-1 decreased with the increases of MW and the sulfur content. On one hand, we suspect that the sulfation modification of curdlan may disturb the hydrophobic or hydrogen bonding resulting in the single helix and random structures, which improve the solubility of the compound. Although the single helix and random structures are active conformations (Kataoka et al., 2002), the high sulfur content may reduce the compound interacting with the groove in dectin-1, and as a result, the binding affinity is weaker than the compound with low sulfur content. On the other hand, it is possible that the increases of MW and sulfur content may cause the increase of steric hindrance. The glucans stereometrically interfere with each other, resulting in less dectin-1 molecules become bound, and this may be the reason that CS1 showed lowest binding affinity (RU) at the highest concentration tested, which agrees with the hypothesis that at too high concentrations, β-1,3-glucan with β-1,6-side-chain branches showed low avidity with dectin-1 (Sonck, Stuyven, Goddeeris, & Cox, 2010). Because of the poor solubility, curdlan was not tested together with curdlan sulfate in SPR analysis assay, thus the difference of binding affinity to dectin-1 between curdlan and curdlan sulfate was not compared, which may require further investigation.

Beside of dectin-1, we also investigated if curdlan sulfate can interact with TLR2. It was demonstrated that dectin-1 could synergize with TLR2 to regulate Syk kinase-dependent cytokine induction, such as TNF-a (Ferwerda et al., 2008; Rogers et al., 2005).

Fig. 7C (upper) indicated high affinity interaction between CSIII and dectin-1 at 800 μg/mL, but could not bind to TLR2 as shown in Fig. 7C (lower). In contrast, LPS at the concentration of 100 μg/mL, which was used as a control in this assay, could interact partly with TLR2, but little binding to dectin-1, which agreed with the results of previous report that LPS might bind to TLR2 although the major receptor is TLR4 (Xu, Yasuda, Tsuruta, Mizuno, & Ashida, 2011). These results demonstrated the sulfation modification of curdlan did not change its primary receptor.

4. Conclusion

In conclusion, curdlan sulfate was demonstrated to have significant activity on macrophages activation and BMDCs maturation through the binding of curdlan sulfate with the primary receptor dectin-1 on cell surface. The immunomodulating activity of curdlan sulfate was similar to that of curdlan in our study. Besides, the combination of CS3 and LPS did not show synergistic effects on the activation of macrophages. Curdlan sulfate induces cytokines production and immune-related molecules expression without any cytotoxicity, and it can be used *via* oral administration or *i.v.* injection, as it is soluble, supporting the notion that curdlan sulfate is beneficial for immunotherapy. These data highlight the ability of non-TLR receptors to bridge innate and adaptive immunity and suggest that dectin-1 agonists may constitute useful adjuvant for immunotherapy and vaccination.

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