Stimulation of RAW264.7 macrophages by sulfated *Escherichia coli* K5 capsular polysaccharide *in vitro*

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Abstract. The aim of the present study was to explore the immunomodulatory effects of sulfated K5 polysaccharide derivatives on RAW264.7 macrophage cells, and to further elucidate the structure-activity relationship. In the present study, chemically sulfated polysaccharides were derived from *Escherichia coli* K5 capsular polysaccharide (K5PS), and molecular weight determination, sugar analysis, and other physical and chemical characterizations were performed on the derived polysaccharides. Enzyme-linked immunosorbent assay and reverse transcription-polymerase chain reaction analyses demonstrated that K5-OS_2 stimulated murine RAW264.7 macrophage cells to release TNF-α and IL-1β proinflammatory cytokines. K5-OS_2 also induced the expression of inducible nitric oxide synthase iNOS, which is responsible for the production of nitric oxide. In addition, K5-OS_2 markedly induced macrophage-mediated cytotoxicity against cancer cells and promoted the phagocytic activity of the RAW264.7 cells. Therefore, K5-OS_2 activated macrophages and acted as a potent immunomodulator. Observations of the present study also indicated that sulfation modification enhanced the immune-enhancing activity of K5PS, and that the high sulfation in the O-position of K5PS may be required for the immunomodulatory activities of the *Escherichia coli* K5 capsular polysaccharide.

Introduction

The *Escherichia coli* K5 capsular polysaccharide (K5PS) is an acidic polysaccharide with a repeating disaccharide unit consisting of β-D-glucuronic acid (GlcA) and α-D-acetylglucosamine (GlcNAc) of (→4) GlcA (1→4) GlcNAc (1→). K5PS has the same carbohydrate backbone as heparosan, which is a precursor in the biosynthesis of heparin and heparan sulfate (1). The K5 polysaccharide exhibits low immunogenicity in humans by acting as a molecular camouflage, which can facilitate bacterial infection of mammals by evading the immune response (2). It is well known that sulfation improves the biological activity of polysaccharides. Several studies have found that sulfated polysaccharides have numerous and marked biological activities (3-5). Previous studies have demonstrated that sulfated modifications of the K5 polysaccharide lead to the synthesis of heparin-like compounds, which exhibit different biological properties, including anticoagulant/antithrombotic, anti-inflammatory, anticancer and antiviral activities (6). The epimerized N,O-sulfated heparosan and O-sulfated heparosan exhibit potential anti-inflammatory activity without the occurrence of bleeding as a side effect (7). These two sulfated heparosan derivatives also inhibit the replication of different HIV-1 strains (8). Therefore, chemical modifications, including partial or per-sulfonation can be applied to obtain more active sulfated polysaccharides.

To the best of our knowledge, various polysaccharides, particluarly following sulfation modification, have immunoregulatory activities, which can be used in healthcare, nutrition and medicine (9,10). Heparin is a known anti-coagulant with a weak direct immunosuppressive action *in vitro* and *in vivo* (11). The inhibitory effects of heparin on human lymphoproliferative response and natural killer (NK) cytotoxicity have been reported in previous studies (12,13). Heparin has been reported to have potential therapeutic immunomodulatory effects during severe sepsis (14). However, heparan sulfate, which is expressed on the cell surface, extracellular matrix and basement membrane, contributes to the immune system by facilitating leukocyte development, leukocyte migration, immune activation and inflammatory processes (15). Given the structural similarities between sulfated K5 derivatives, heparin and heparan sulfate, the K5 polysaccharide with sulfated modifications may also have immunomodulatory properties. However, the effect of sulfated K5 polysaccharides on the immune system remain to be fully elucidated.

The aim of the present study was to examine the effects of sulfated K5 polysaccharide derivatives on RAW264.7 macrophage cells, and to examine macrophage phagocytosis, the release of nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β). The gene expression levels of TNF-α, IL-1β, and inducible nitric oxide synthase (iNOS)

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were also examined. Morphological changes were analyzed to investigate the potential roles of K5 polysaccharide in human immunity following sulfated modifications. The immunomodulatory activities of sulfated K5 polysaccharide derivatives on murine RAW264.7 macrophage cells were investigated to assist in the development of novel pharmaceutical products. In addition, the presents study aimed to elucidate the structure-activity associations among sulfated polysaccharides.

Materials and methods

Materials and chemicals. The pyridine-sulfotrioxyde complex, tetrabutylammonium hydroxide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS; *E. coli* 0111:B4) and trypsin were from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin (BSA) and fetal bovine serum (FBS) were obtained from Gibco Life Technologies (Carlsbad, CA, USA). All other chemicals and solvents used were of analytical reagent grade and obtained from Sinopharm Chemical Reagent Co, Ltd. (Shanghai China). The enzyme-linked immunosorbent assay (ELISA) kits for IL-1β, TNF-α were obtained from Shanghai Fu Sheng Industrial Co., Ltd. (Shanghai, China).

Preparation of the K5PS and sulfated modification. The capsular K5PS was produced from *E. coli* strain 010:K5:H4 fermentation and purification from the culture supernatant, as described previously (2,16).

The sulfation reagent was prepared in two concentrations with ratios of K5PS:pyridine-sulfotrioxyde complex at 1:0.5 and 1:2.5, respectively. Briefly, K5PS (1 g) was suspended in anhydrous *N,N*-dimethylformamide (25 ml), followed by the addition of the sulfation reagent. The mixture was maintained at room temperature for 18 h with continuous stirring. Subsequently, the mixture was cooled and recovered by precipitation with NaCl-saturated acetone (20 ml). Finally, the sulfated polysaccharide was dialyzed against distilled water, and was freeze-dried as samples termed K5-OS_1 and K5-OS_2. To obtain the N-deacetylated/N-sulfated K5PS (K5-NS), K5PS (1 g) was dissolved in 2 M NaOH and incubated for 24 h at 60˚C. The solution was then warmed to 40˚C and added, in a single step to the sodium carbonate and pyridine-sulfotrioxyde complex for 4 h, and incubated for an additional hour at the same temperature. The K5-NS was run through a cation exchange column (IR-120 H+; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 10˚C and was neutralized with 15% tetrabutylammonium hydroxide in water. Following freeze-drying, the sample was dissolved in *N,N*-dimethylformamide (40 ml), and the sulfation reagent was added to synthesize N,O-sulfated K5PS (K5-N, OS), as described above.

Lipopolysaccharode (LPS)-free samples of K5 and sulfated K5PS were prepared using endotoxin removal resin (1.5 ml; Genscript USA, Inc., Piscataway, NJ, USA). Endotoxin was removed, resulting in levels <0.1 EU/ml, as indicated using a Limulus Amebocyte Lysate assay (Xiamen Limulus Reagent Factory, Xiamen, China), according to the manufacturer's protocol.

Characterization of sulfated K5 derivatives. Molecular weight determination was performed by gel permeation chromatography using a multi-angle light scattering detector (GPC-MALS; Wyatt Technology Corporation, Goleta, CA, USA). Briefly, the concentration of polysaccharide solution was 3.0 mg/ml in sodium chloride solution. The polysaccharide solution in sodium chloride (200 µl) was detected after being filtered through a 0.2 µm syringe filter. Astra software (Astra V; Wyatt Technology Corporation) was utilized for data acquisition and analysis. The content of uronic acid was estimated using a carbazole method, with D-glucuronic acid as a standard (17). Sulfate content was determined using a barium chloride-gelatin method (18). The degree of sulfation (DS), which indicated the average number of sulfate ester groups on each monosaccharide residue, was calculated based on the sulfur content of the prepared samples (4).

Macrophage activation by sulfated K5PS derivatives in vitro.

Cell culture, morphological observations and assessment of cell viability. The RAW264.7 cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in DMEM supplemented with 10% FBS containing 100 U/ml penicillin and 100 µg/ml streptomycin at 37˚C in a 5% CO_2 incubator. Polymyxin B (50 µg/ml) was added to each well to eliminate LPS contamination. In brief, the cells were seeded at $1x10^5$ cells/ml in a 96-well plate for the MTT assay. After 24 h at 37˚C, different concentrations of sulfated K5 derivatives (1, 10, 50 and 100 µg/ml) were added and the mixtures were cultured for 24 h at 37˚C. Subsequently, the cells were visualized using light microscopy with a Leica DMIL LED microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA; magnification, x400). Following morphological observation, the medium was removed and replaced with fresh medium containing 0.5 mg/ml MTT. The plates were further incubated for 4 h at 37˚C. Following the production of formazan crystals, the crystals were dissolved in DMSO, and the absorbance at 570 nm was measured using a microplate reader (Multiskan GO; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Phagocytosis of sulfated K5 polysaccharide derivative-stimulated RAW264.7 macrophages. The RAW264.7 cells were seeded ($1x10^5$ cells/ml) in a 96-well plate with DMEM medium (10% FBS) and incubated at 37˚C in 5% CO_2 for 24 h. The cells were cultured with the samples at final concentrations of 1, 10, 50 and 100 µg/ml for 24 h at 37˚C. DMEM and 10 µg/ml LPS were used as negative and positive controls, respectively. The stimulated cells were washed twice with phosphate-buffered saline (PBS), and 100 µl of 0.075% neutral red solution was added to each well, followed by incubation for 4 h at 37˚C. Following removal of the unphagocytized neutral red by PBS, cell lysis buffer (acetic acid/ethanol; 1:1) was added to each well at 4˚C for 2 h. The optical density (OD) value of each well was measured at 570 nm using a microplate reader. Cell phagocytosis (%) was calculated as follows: OD_S / OD_C x 100, where OD_S and OD_C represent the OD values of the stimulated and control wells, respectively. The percentage of phagocytosis in the untreated cells was designated as 100%. All experiments were performed in triplicate.

Measurement of NO in the RAW264.7. Cells A total of $5x10^4$ cells/well were seeded into a 96-well culture plate.

Table I. Primer sequences and cycling parameters used for reverse transcription-polymerase chain reaction.

Gene	Primer sequence (5' to 3')	Annealing temperature (˚C)
iNOS	Forward: GAGCGAGTTGTGGATTGTC Reverse: CCAGGAAGTAGGTGAGGG	55
TNF-α	Forward: CACCCTTATTCTCGCTCAC Reverse: CCCGCTTACAGTTCCTCT	53
IL-1β	Forward: CTCGTGCTGTCGGACCCAT Reverse: CAGGCTTGTGCTCTGCTTGTGA	57
GAPDH	Forward: CTCTGCTCCTCCCTGTTC Reverse: CAATCTCCACTTTGCCACT	52

iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor; IL, interleukin.

After 24 h, the cells were pre-incubated with different sample concentrations, as mentioned above. LPS (10 μg/ml) was used as a positive control. The NO concentrations were estimated by calculating the quantity of released nitrite, using Griess reagent, in the cell supernatant. The OD was determined at 540 nm using a microplate reader, with data expressed as the mean ± standard deviation of three experiments.

Measurement of cytokines in the RAW264.7. The levels of TNF-α and IL-1β were determined using ELISA kits, according to the manufacturer's instructions. Briefly, the RAW264.7 cells were cultured for 24 h with different concentrations of the sample, as described above. The supernatants were harvested and standard curves were produced for calculation of the cytokine concentrations using Origin 8.0 (OriginLab Corporation, Northampton, MA, USA).

Gene expression levels of iNOS, TNF-α, and IL-1β using reverse transcription-polymerase chain reaction (RT-PCR). In the present study, RT-PCR was performed to determine changes in the gene expression levels of iNOS, TNF-α and IL-1β. The macrophages were cultured in the presence or absence of the samples for the indicated time-periods. The macrophages were lysed using TRIzol reagent (Invitrogen Life Technologies) and the total RNA was extracted, according to the manufacturer's instructions. Reverse transcription of the RNA was performed using RevertAid First Strand cDNA Synthesis kit (Fermentas, Thermo Fisher Scientific, Inc.). PCR was carried out using a Bio-Rad MyCycler thermal cycler (Bio-Rad Laboratories, Inc.) as follows: 12.5 μl Premix *Taq* (Takara Biotechnology, Inc., Dalian, China), 1 μl template cDNA, 0.5 μl each primer (Shanghai Sangon Biotechnology Co., Ltd., Shanghai, China), and water up to 25 μl. PCR cycling conditions were as follows: 95˚C for 3 min, followed by 95˚C for 45 sec, 55˚C (or 53˚C, 57˚C or 52˚C) for 45 sec, and 72˚C for 45 sec for 35 cycles, and a final extension for 10 min at 72˚C. The PCR product was maintained at 4˚C until further use. PCR products were electrophoresed on a 2% agarose gel and an image was captured using a Bio-Rad Gel Doc XR+ imaging system (Bio-Rad Laboratories, Inc.). Optical density of the bands was quantified using Quantity One 4.6 software (Bio-Rad Laboratories, Inc.).

The sequences of the primers used are presented in Table I.

Measurement of cytotoxicity induced by sulfated K5PS-treated macrophages. Macrophage-mediated tumor cell cytotoxicity was measured, as described previously (19). The sulfated K5PSs-treated macrophages, which were treated for 24 h followed by PBS washing, were co-incubated with cancer cells ($1.0x10^4$ cells/well murine B16 melanoma cells or human cervical cancer HeLa cells; Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) for 24 h at a cancer cell:macrophage ratio of 1:10. The viability of the cancer cells was then determined using an MTT assay, to measure macrophage-induced cytotoxicity.

Table II. Chemical composition of the K5 samples.

Sample	Uronic acid (%)	Sulfate (%)	DS	Mw (Da)
K5	33.14	0	0	$7.22x10^4$
K5-OS_1	32.95	0.21	0.01	$6.58x10^4$
K5-OS_2	27.33	2.87	0.16	$6.89x10^4$
K5-NS	26.92	5.56	0.34	$9.31x10^3$
K5-N,OS	26.67	6.01	0.38	$1.02x10^4$

DS = (1.62 x S) / (32 - 1.02 x S). DS, degree of sulfate substitution; S, sulfate; Mw, molecular weight; K5, K5 capsular polysaccharide; K5-OS_1, 1:0.5 K5PS:pyridine-sulfotrioxyde complex; K5-OS_2, 1:2.5 K5PS:pyridine-sulfotrioxyde complex; K5-NS, N-deacetylated/N-sulfated K5PS; K5-N, OS, N,O-sulfated K5PS.

Statistical analysis. The results are presented as the mean ± standard deviation. One-way analysis of variance for was performed using SPSS version 19.0 (IBM SPSS, Armonk, NY, USA). Tukey's method for multiple comparisons among groups was used to determine significant differences. $P<0.05$ was considered to indicate a statistically significant differences.

Results

Characterization of sulfated K5PSs. The chemical compositions of the K5-OS_1, K5-OS_2, K5-NS, and K5-N,OS samples are presented in Table II. K5-OS_1 and K5PS contained similar total sugar (uronic acid) contents. Compared with K5-OS_1, the total sugars in the sulfation derivative K5-OS_2, K5-NS and

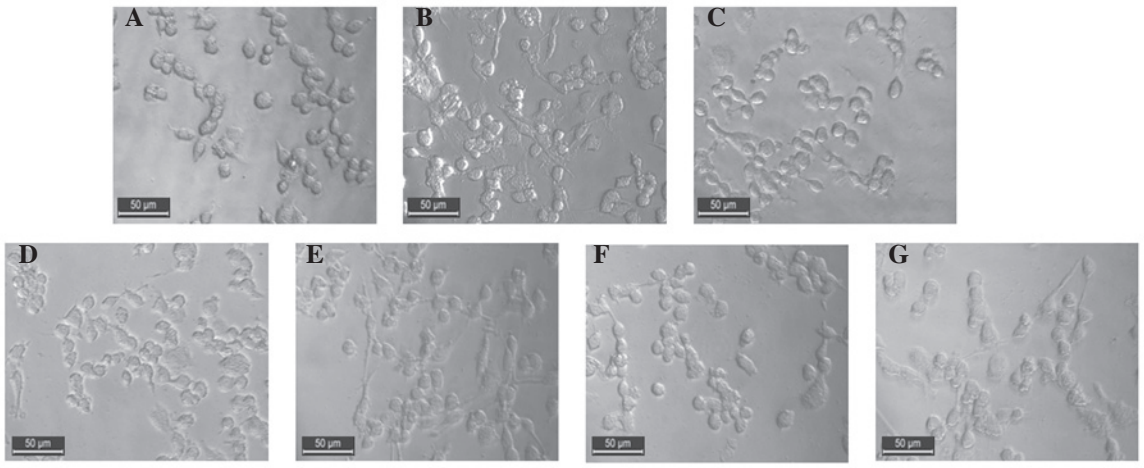

Figure 1. Morphology of RAW 264.7 cells 24 h after treatment with (A) medium, (B) 10 μg/ml lipopolysaccharide, (C) 100 μg/ml K5, (D) 100 μg/ml K5-OS_1 (E) 100 μg/ml K5-OS_2, (F) 100 μg/ml K5-NS, and (G) 100 μg/ml K5-N,OS (magnification x400). K5, K5 capsular polysaccharide; K5-OS_1, 1:0.5 K5PS:pyridine-sulfotrioxyde complex; K5-OS_2, 1:2.5 K5PS:pyridine-sulfotrioxyde complex; K5-NS, N-deacetylated/N-sulfated K5PS; K5-N, OS, N,O-sulfated K5PS.

K5-N,OS samples decreased following modification. The sulfate content and DS of the sulfated K5PSs are listed in decreasing order, as follows: K5-N,OS>K5-NS>K5-OS_2>K5-OS_1. This finding indicated that the sulfation of different degrees had been successful. As shown in Table II, the molecular weights following sulfation was lower than that of the K5PS, which was possibly due to hydrolysis of the dominant chains during the sulfation process.

Effects of sulfated K5PSs on the growth of RAW264.7 cells. To examine whether sulfated K5PSs were capable of modulating the functional activation of macrophages, the RAW264.7 cells were treated with the samples and morphological changes were observed after 24 h (Fig. 1). When the RAW264.7 cells were cultured in the medium (Fig. 1A), the majority of the cells were observed to exhibit a circular morphology, whereas the morphologies of the cells cultured in the presence of LPS were altered (Fig. 1B). When the cells were co-cultured with K5PS and the sulfated derivatives (Fig. 1C-G), morphological alterations associated with macrophage activation were observed after 24 h.

To determine whether the sulfated K5PSs were cytotoxic to RAW264.7 cells, the present study analyzed cell viability at various concentrations of K5 and sulfated K5PSs (1, 10, 50 and 100 μg/ml) using an MTT assay. Following 24 h of incubation with varying concentrations, the A_{570} values of each group were assessed (Table III). The A_{570} values of all samples at concentrations of 100 μg/ml were significantly higher, compared with those in the cell control group (P<0.05). Furthermore, a significant difference (P<0.05) was observed between the A_{570} values of the 50 and 100 μg/ml concentrations of K5-OS_2 and those of the other groups. No cytotoxic effects were observed of K5PS or the sulfated derivatives on the RAW264.7 cells.

Effects of sulfated K5PSs on phagocytic activity. Based on the results described above, the present study subsequently investigated the effect of K5, K5-OS_1, K5-OS_2, K5-NS and K5-N,OS on the phagocytic activity of RAW 264.7 cells. Neutral red solution is commonly used in phagocytosis assays due to its ease of manipulation and determination through examination of OD values (20,21). The effects of the neutral red-ingested macrophages in the different treatment groups on phagocytosis are shown in Fig. 2. During the single period of polysaccharide stimulation, the phagocytic activity of the K5-OS_2 group was significantly higher, compared with the activities in the untreated control and remaining treatment groups (P<0.05), exhibiting a dose-dependent pattern. K5-OS_2 increased phagocytosis between 22.8±3.8 and 46.0±4.6%. Compared with the control group, K5-N,OS (100 μg/ml) and LPS (10 μg/ml) increased the number of macrophages exhibiting neutral red ingestion, which had higher OD values. The K5-OS_1 and K5-NS groups were unable to individually augment the phagocytic activity of the RAW264.7 cells by 105.8±3.1% and 101.7±7.2%.

Effects of sulfated K5PSs on the production of NO in RAW264.7 macrophages. The effects of the sulfated K5PSs on the production of NO in the RAW264.7 macrophages were investigated using the Griess reaction in the culture medium (21). The production of NO was low in the untreated RAW264.7 cells, however, the production increased moderately with the sulfated K5PSs, and increased markedly with LPS. As shown in Fig. 3, when the RAW264.7 cells were treated with the four polysaccharides, synthesized with different sulfation patterns, the production of NO increased in a dose-dependent manner (P<0.05). However, K5PS had no effect on inducting the production of NO. K5-N,OS minimally increased the production of NO at concentrations of 1 or 10 μg/ml, and moderately stimulated NO at higher concentrations. By contrast, K5-OS_2 and K5-OS_1 were highly active and stimulated levels of macrophage NO production, which were comparable to that in the control. The production of NO by the RAW264.7 cells incubated with K5-OS_2 or K5-OS_1 at concentrations of 100 μg/ml for 24 h were 249.2±11.2 and 221.2±17.1% of the control value, respectively (P<0.05; Fig. 3).

Effects of sulfated K5PSs on macrophage production of TNF-α and IL-1β. To determine the effect of sulfated K5PS-activated RAW264.7 cells on the expression of cytokines, the culture

Table III. Macrophage viability changes in each group in the presence of polysaccharide stimulation.

Concentration (μg/ml)	A_{570} value				
	K5	K5-OS_1	K5-OS_2	K5-NS	K5-N,OS
0 (control)	0.653±0.004	0.656±0.003	0.657±0.007	0.651±0.002	0.652±0.003
1	0.643±0.004	0.641±0.010	0.674±0.003	0.629±0.013	0.629±0.009
10	0.652±0.018	0.653±0.002	0.685±0.003	0.629±0.003	0.669±0.009
50	0.662±0.005	0.665±0.012	0.804±0.006[a]	0.664±0.003	0.674±0.030
100	0.695±0.014[a]	0.712±0.007[a]	0.842±0.017[a]	0.693±0.008[a]	0.711±0.007[a]

[a]P<0.05, compared with the control group. K5, K5 capsular polysaccharide; K5-OS_1, 1:0.5 K5PS:pyridine-sulfotrioxyde complex; K5-OS_2, 1:2.5 K5PS:pyridine-sulfotrioxyde complex; K5-NS, N-deacetylated/N-sulfated K5PS; K5-N, OS, N,O-sulfated K5PS.

Table IV. Effects of sulfated K5PSs on cytokine production by stimulated RAW264.7 cells.

Sample	Concentration (μg/ml)	Production (pg/ml)	
		TNF-α	IL-1β
Control	0	871.0±13.3	96.1±2.0
LPS	10	974.0±15.0[a]	117.2±1.7[a]
K5	1	867.3±16.5	97.0±3.1
	10	877.3±12.1	97.7±2.1
	50	879.3±22.3	99.6±3.9
	100	908.4±23.6	102.4±1.6
K5-OS_1	1	877.3±27.0	113.0±0.5[a]
	10	918.5±57.9	114.1±1.0[a]
	50	922.6±57.2	114.2±0.5[a]
	100	935.3±26.7	114.1±0.4[a]
K5-OS_2	1	1,042.3±31.9[a]	117.4±1.4[a]
	10	1,045.8±49.3[a]	120.0±1.7[a]
	50	1,111.8±72.1[a,b]	121.6±1.8[a]
	100	1,180.4±34.3[a,b]	123.1±0.3[a]
K5-NS	1	844.3±46.0	96.0±2.1
	10	895.4±12.0	97.8±3.0
	50	894.8±17.0	99.9±4.9
	100	949.5±18.2	100.4±2.7
K5-N,OS	1	1,049.2±34.2[a]	98.3±1.5
	10	1,055.7±33.6[a]	103.2±3.5
	50	1,095.8±14.5[a]	108.0±1.9[a]
	100	1,161.8±113.4[a,b]	120.5±3.5[a]

[a]P<0.05 compared with control, [b]P<0.05 compared with LPS group. TNF, tumor necrosis factor; IL, interleukin; K5, K5 capsular polysaccharide; LPS, lipopolysaccharide; K5-OS_1, 1:0.5 K5PS:pyridine-sulfotrioxyde complex; K5-OS_2, 1:2.5 K5PS:pyridine-sulfotrioxyde complex; K5-NS, N-deacetylated/N-sulfated K5PS; K5-N, OS, N,O-sulfated K5PS.

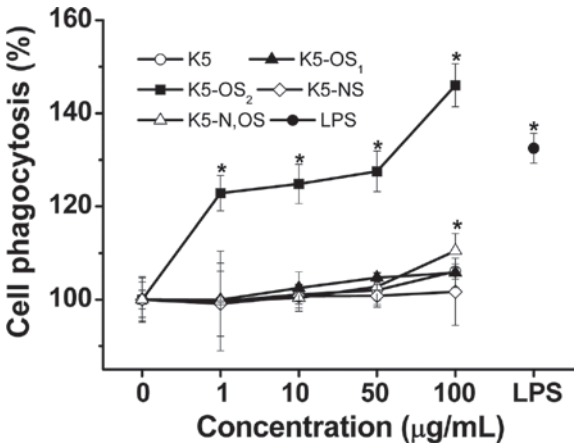

Figure 2. Effects of sulfated K5PSs on the phagocytosis of RAW 264.7 cell, determined using a neutral red uptake assay. Each value is expressed as the mean ± standard deviation of three independent experiments. *P<0.05, compared with the untreated control group. K5, K5 capsular polysaccharide; K5-OS_1, 1:0.5 K5PS:pyridine-sulfotrioxyde complex; K5-OS_2, 1:2.5 K5PS:pyridine-sulfotrioxyde complex; K5-NS, N-deacetylated/N-sulfated K5PS; K5-N, OS, N,O-sulfated K5PS; LPS, lipopolysaccharide.

supernatants were collected following 24 h of exposure, and the levels of TNF-α and IL-1β were determined using ELISA kits. As shown in Table IV, among the four sulfated K5PS groups, K5-OS_2 significantly increased cytokine release from the macrophages at every concentration, whereas K5-OS_1 only increased the level of IL-1β between 113.0±0.5 and 114.1±0.4 pg/ml (P<0.05). In addition, K5-N,OS also increased the release of IL-1β from the macrophages in a dose-dependent manner and markedly increased the production of IL-1β up to 120.5±3.5 pg/ml at high concentrations. The RAW264.7 cells treated with K5-N,OS produced considerably higher concentrations of TNF-α, with levels reaching ~1161.8±113.4 pg/ml at the 100 μg/ml concentration (P<0.05). By contrast, no significant increases in TNF-α and IL-1β were observed in the K5 and K5-NS groups, at any concentration, in RAW264.7 cells.

Effects of sulfated K5PSs on the mRNA levels of iNOS, TNF-α and IL-1β. Activated macrophages are known to release immune factors, including NO, TNF-α and IL-1β (21-23). To assess the activation of macrophages by the sulfated K5PSs in the present study, RT-PCR analysis was performed to evaluate the mRNA expression levels of iNOS, TNF-α and IL-1β (Fig. 4A). Following treatment of the samples at 100 μg/ml concentrations for 24 h, the RAW264.7 cells expressed mRNA

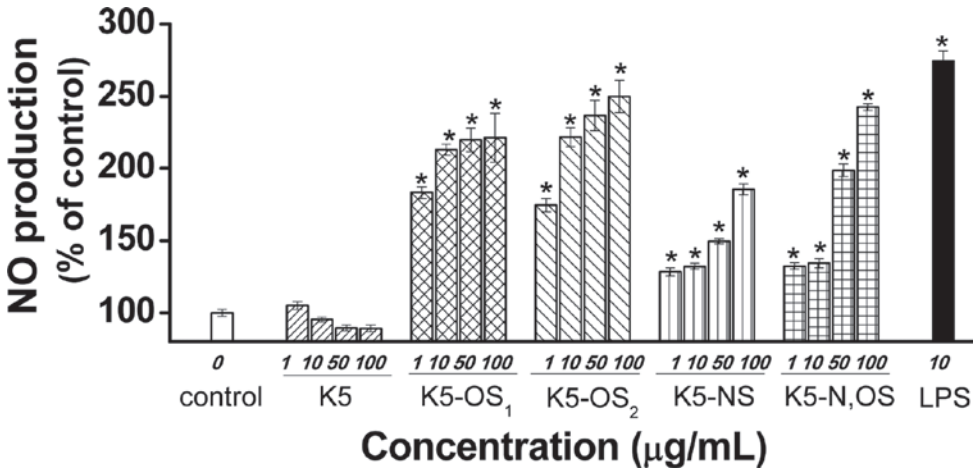

Figure 3. Effects of sulfated K5PSs on the production of NO in RAW 264.7 cells. NO production was measured using a Griess reaction assay and data are expressed as a percentage of the untreated control cells, and as the mean ± standard deviation of three independent experiments. The differences between the control group and treatment groups were determined using one-way analysis of variance *P<0.05, compared with the control. NO, nitrous oxide; K5, K5 capsular polysaccharide; K5-OS_1, 1:0.5 K5PS:pyridine-sulfotrioxyde complex; K5-OS_2, 1:2.5 K5PS:pyridine-sulfotrioxyde complex; K5-NS, N-deacetylated/N-sulfated K5PS; K5-N, OS, N,O-sulfated K5PSl; LPS, lipopolysaccharide.

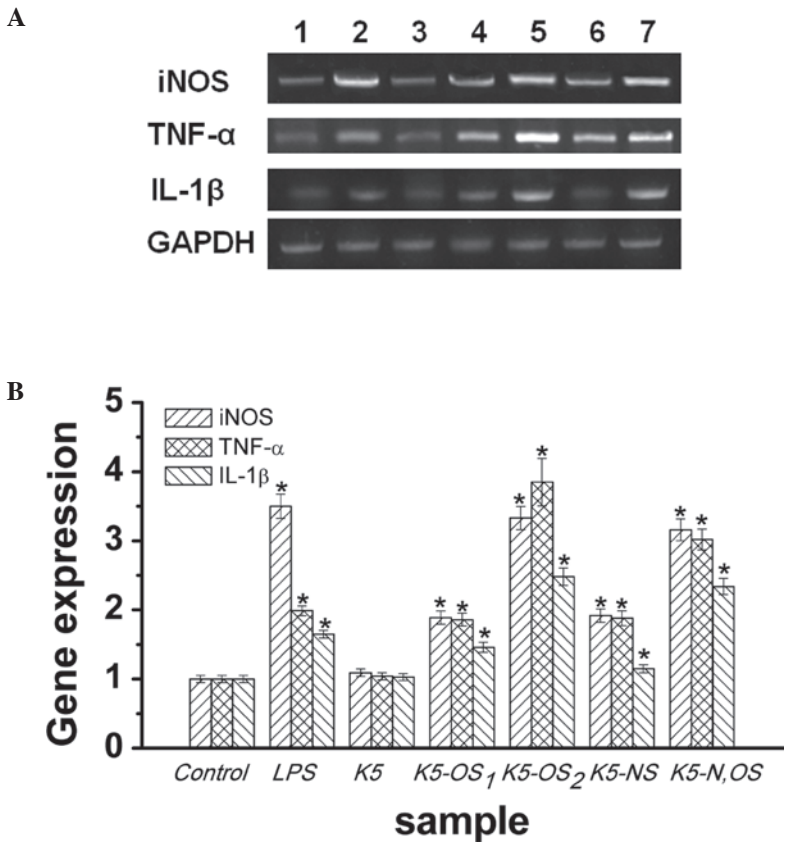

Figure 4. Effects of sulfated K5PSs on the mRNA expression levels of iNOS, TNF-α, and IL-1β. RAW264.7 cells were incubated for 24 h with samples (100 μg/ml) or LPS (10 μg/ml). (A) mRNA levels were determined using reverse transcription-polymerase chain reaction.. Lanes 1-7 indicate the control, LPS, K5, K5-OS_1, K5-OS_2, K5-NS, and K5-N,OS groups, respectively. (B) Semi-quantification of the mRNA expression levels of iNOS, TNF-α, and IL-1β, as the ratio of densitometric measurement with that of the internal standard (GAPDH). Significance was determined using one-way analysis of variance (*P<0.05, compared with the control group). Data are expressed as the mean ± standard deviation. iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor; IL, interleukin; K5, K5 capsular polysaccharide; K5-OS_1, 1:0.5 K5PS:pyridine-sulfotrioxyde complex; K5-OS_2, 1:2.5 K5PS:pyridine-sulfotrioxyde complex; K5-NS, N-deacetylated/N-sulfated K5PS; K5-N, OS, N,O-sulfated K5PS; LPS, lipopolysaccharide.

encoding the genes for iNOS, TNF-α and IL-1β, which are established markers of macrophage activation (Fig. 4A). As estimated using densitometric measurement, K5-OS_2 stimulated the expression of iNOS, TNF-α and IL-1βin macrophages at three times the level observed in the normal control (Fig. 4B). Compared with the control, treatment with K5-N,OS by itself significantly increased the mRNA levels of iNOS, TNF-α and IL-1β by 216, 202, and 134% (Fig. 4B), which were comparable

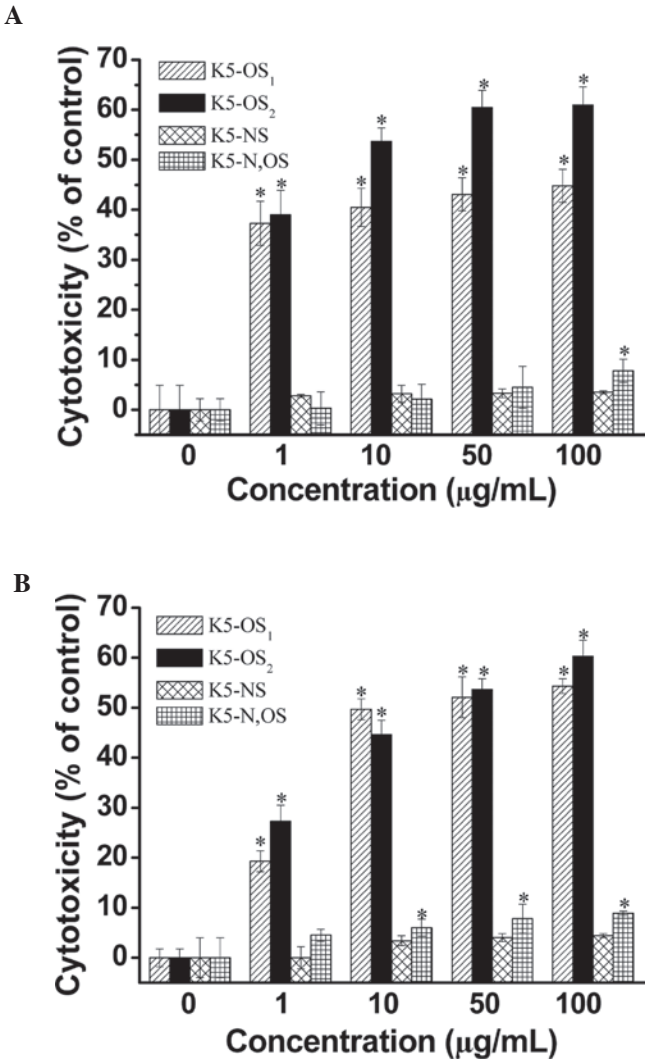

Figure 5. Cytotoxicity of activated RAW264.7 cells against (A) B16 cells and (B) Hela cells. RAW264.7 cells were stimulated with sulfated K5PSs to generate cytotoxic macrophages. Cytotoxicity was evaluated against the specified target cells at an effector-target ratio of 10. Data are presented as the mean ± standard deviation of triplicate experiments. *P<0.05, compared with the inactivated-macrophage control group. K5, K5 capsular polysaccharide; K5-OS_1, 1:0.5 K5PS:pyridine-sulfotrioxyde complex; K5-OS_2, 1:2.5 K5PS:pyridine-sulfotrioxyde complex; K5-NS, N-deacetylated/N-sulfated K5PS; K5-N, OS, N,O-sulfated K5PS.

to the levels observed in the LPS treatment group. By contrast, K5 had no significant effect on mRNA expression, which was consistent with the ELISA assay data.

Effects of sulfated K5PSs on macrophage-mediated cytotoxicity against cancer cells in vitro. The immunomodulatory effect of polysaccharides is suggested to occur in cancer via macrophage-mediated cytotoxicity against cancer cells (24). Therefore, the present study examined the cytotoxicity of activated RAW264.7 cells against tumor cells. As shown in Fig. 5, K5-OS_2 and K5-OS_1 enhanced macrophage-induced cytotoxicity against the B16 (Fig. 5A) and Hela (Fig. 5B) tumor cell lines in the concentration range of 1-100 μg/ml. The macrophage-induced cytotoxicity of K5-OS_2 was increased in a dose-dependent manner, with levels reaching ~62%, compared with the control, at the 100 μg/ml concentration (P<0.05). K5-N,OS only enhanced cytotoxicity (7.8%) against B16 cell lines at the highest dose (P<0.05; Fig. 5A). However, K5-N,OS improved the cytotoxicity against Hela cells in the concentration range of 10-100 μg/ml (P<0.05; Fig. 5B). The results also revealed a significant difference between the K5-OS_2 and the K5-NS, K5-N,OS, and control groups at all concentrations assessed (P<0.05). However, no significant difference was observed between the K5-NS group and the control at any concentration (P>0.05). Similarly, unmodified K5PS had no effect on macrophage-mediated cytotoxicity against cancer cells (data not shown).

Discussion

To the best of our knowledge, macrophages are the first cells to recognize infectious agents. Macrophages are involved in specific and non-specific immune reactions (25). Therefore, the identification of agents,which can modulate macrophages is of significant interest. A number of polysaccharides have been reported to exhibit beneficial pharmacological effects due to their ability to modulate macrophage function (26). In the present study, K5PS was prepared from *E. coli*, and sulfated derivatives of K5PS with different sulfation patterns were prepared via chemical modifications, the effects of which

were investigated in mouse macrophage RAW264.7 cells, a model, which is closer to humans (21). Based on the results of the MTT assay and morphological observations, the present study demonstrated that sulfated K5PSs induced macrophage activation, without cytotoxicity, in the RAW264.7 cells.

In addition, the sulfated K5PSs significantly enhanced the production of NO in the RAW264.7 cells in a dose-dependent manner *in vitro*. The release of NO from macrophages is an intracellular messenger molecule in the nonspecific immune response (25,27). The release of NO has been suggested to be important in the immune response (28). Therefore, sulfated K5PSs may be an immune mediator/modulator with multiple biological functions, including macrophage-mediated activity. Activated macrophages are considered to be associated with cytokine release. Cytokines are signaling molecules, which control homeostasis in organisms by the regulation of cell differentiation, proliferation and apoptosis, and also regulate defense functions, including immune responses (29). TNF-α, produced by activated macrophages, T lymphocytes and NK cells, functions as a key cytokine in immune and inflammatory reactions (30). IL-1β is one of the most important cytokines that facilitates activated macrophage release and functions as a mediator of the immune system (29). In the present study, K5-OS_2 and K5-N,OS were observed to markedly stimulate RAW264.7 cells to release TNF-α and IL-1β. Therefore, K5-OS2 and K5-N,OS may be investigated as novel immune-stimulants for drugs in the future (28). K5-OS_2 and K5-N,OS also upregulated the expression levels of iNOS, TNF-α and IL-1β mRNA, which may explain the immunostimulatory properties of K5-OS_2 and K5-N,OS. Our previous study also confirmed that the stimulation of macrophages by sulfated polysaccharides was superior to the stimulation provided by unmodified polysaccharides (5).

The production of NO by activated macrophages can lead to cytostatic and cytotoxic activities on malignant cells (31). The results of the present study revealed that K5-OS_1 and K5-OS_2 markedly induced macrophage-mediated cytotoxicity against cancer cells. Therefore, macrophages activated by K5-OS_1 and K5-OS_2 may exert tumoricidal activity through NO-dependent pathways (32). K5-OS_2 promoted phagocytosis, which is the first step in the macrophage response to invading microorganisms, followed by the activation of phagocytosis, which elevates the innate immune response (33,34). The results of the present study indicated that K5-OS_2 may have indirect antitumor activity by improving immunologic function.

Previous investigations have demonstrated that polysaccharides have several bioactivities and that appropriate molecular modification or structure reform may lead to the generation or enhancement of activity in polysaccharides (5). Sulfated modification of polysaccharides is one of the commonly used modification methods. Sulfated polysaccharides exert potent biological properties, which are relative to the degree of substitution and the position of sulfated groups (4). Therefore, the DS of polysaccharides may be an important parameter that affects bioactivities. In the present study, K5-OS_2 exhibited the most marked activity, which can be attributed to a higher DS. K5-OS_1 had a lower DS, which indicated that the sulfate esters were important in its biological activity (4,35), however, previous evidence suggests that the position of the sulfate substituent is important for bioactivity (27), consistent with the present study. K5-NS, with a higher DS at the N-position, exhibited minimal stimulation of the RAW264.7 cells *in vitro*, whereas K5-N,OS induced macrophage formation and exhibited immunoregulation activity. Comparison between the structures and activities of K5-NS and K5-N,OS indicated that the sulfate substitution position was one of the key structural factors that determined the efficacy of RAW264.7 macrophage stimulation *in vitro* (36). Sulfation in the O-position of the K5 polysaccharide appeared to be more effective, compared with sulfation in the N-position.

In conclusion, sulfation modification was observed to significantly enhance the macrophage immunomodulatory activity of *E. coli* K5PSs. High levels of sulfation in the O-position of K5PSs may be required to produce immunomodulatory activities. The results of the present study also suggested that K5-OS_2 may be a promising novel immunopotentiator.

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