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Immunomodulatory activity of heparan sulfate mimetics from *Escherichia coli* K5 capsular polysaccharide *in vitro*

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ABSTRACT

Escherichia coli K5 capsular polysaccharide (K5PS) has been used as starting material to synthesize heparan sulfate (HS) mimetics with various biological properties. This study determined the immunomodulatory activities of K5PS through the sulfation process. The immunomodulatory effects of sulfated K5 polysaccharide derivatives were evaluated *in vitro* on murine macrophages and lymphocytes. Results indicated that HS mimetics with high sulfation in the *O*-position stimulated cell proliferation of macrophages and lymphocytes, significantly enhanced cytokine secretion and upregulated the cytotoxicity of NK cells. This study suggests that high sulfation in *O*-position of HS is required for the immunomodulatory activities.

1. Introduction

Heparan sulfate (HS) is a member of the glycosaminoglycan family of polysaccharides that are a ubiquitous class of sulfated polysaccharides. HS is present in almost all tissues on the cell surfaces and extracellular matrix in the form of HS proteoglycans (HSPGs), in which its polysaccharide chains are covalently attached to the core proteins. It is involved in diverse biological processes, such as development, tissue homeostasis, angiogenesis, inflammation, and immunity (Simon Davis & Parish, 2013). The major function of the HS chains of HSPGs, a major one is their ability to interact with a wide variety of bioactive molecules, such as growth factors, proteases cytokines, and chemokines (Shute, 2012). HS polysaccharides are considered inhibitors or promoters in immune response (Chen, Wu, & Wen, 2008). The interaction between HS and immune cells enhances the processes of recognizing and arresting antigen, moving into and out of immune organs, and promoting the proliferation of lymphocytes. However, some HS polysaccharides function as immunosuppressors by blocking proinflammatory cytokines, suppressing activation of complements, and inhibiting migration and adhesion of immune cells through their interactions with chemokines (Chen, Wu, & Wen, 2008; Simon Davis & Parish, 2013). These interactions and the corresponding pathophysiological functions can be modulated by exogenous HS mimetics (Li, Zheng, Li, & Ma, 2012; Sheng, Oh, Chang, & Hsieh-Wilson, 2013).

Escherichia coli K5 capsular polysaccharide (K5PS) has been used as the starting material to synthesize HS analogs with various biological properties because of its similar carbohydrate backbone as the HS biosynthetic precursor. Chemically and/or enzymatically sulfated K5PSs possess antiproliferative, antiangiogenic, antimetastatic, and antiviral activities (Li et al., 2013). However, neither the immunomodulatory activities of the sulfated K5PSs nor their functional mechanism is clearly elucidated. The possibility that sulfated K5PSs will disturb the function of HS in immune response has not yet been investigated, although many sulfated polysaccharides have various effects on innate immune and complement system.

To clarify the immunoregulatory properties of sulfated K5PSs, this study examined the effect of different concentrations and sulfation patterns of sulfated K5PSs on macrophages and spleen lymphocytes *in vitro*. It aimed to elucidate the structure–activity relationships among the sulfated K5PSs, and further develop a new immunopotentiator.

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2. Materials and methods

2.1. Materials and reagents

Pyridine–sulfotrioxyde complex, tetrabutylammonium hydroxide, trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS, *E. coli* 0111:B4), and concanavalin A (ConA) were purchased from Sigma, USA. RPMI 1640 medium, Dulbecco's modified Eagel's (DMEM) medium, bovine serum albumin (BSA), and fetal bovine serum (FBS) were obtained from Gibco, USA. All other chemicals and solvents used were of analytical reagent grade and obtained from Sinopharm Chemical Reagent Co., Ltd., China. The enzyme-linked immunosorbent assay (ELISA) kits for interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), immunoglobulin G 1a (IgG 1a), IgG 2b, interferon-gamma (IFN-γ), and interleukin-2 (IL-2) were obtained from Shanghai Fu Sheng Industrial Co., Ltd., China.

2.2. Animals

Imprinting control region (ICR) mice (male, 20.0 ± 2.0 g) were purchased from the Laboratory Animal Center of Zhejiang Province, China (the license number: SCXK (Zhe) 2008-0033). During the experiment the mice were kept under standard laboratory conditions. All the experimental procedures were approved by the Animal Care Committee of Jiangnan University of China.

2.3. Cell lines

YAC-1 (Mouse lymphoma cell line) and RAW 264.7 cells (RAW cells, mouse macrophage cell line) were obtained from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. Cells were cultured in RPMI 1640 or DMEM supplemented with 10% FBS containing 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37 °C under humidified air with 5% CO_2.

2.4. Preparation of K5 derivatives

The capsular K5 polysaccharide was produced by *E. coli* strain 010:K5:H4 fermentation and purified from the culture supernatant, as described previously (Wang et al., 2010b; Zhang et al., 2012).

Four classes of sulfated derivatives with different degrees of *N*- or *O*-sulfation (Fig. 1) were synthesized as previously described (Leali et al., 2001). Briefly, K5-OS_1 and K5-OS_2 were obtained by direct *O*-sulfation of a single batch of K5 polysaccharide added with different concentrations of pyridine–sulfotrioxyde complex. The sample with a relatively higher degree of *O*-sulfation was named as K5-OS_2. Another one with a relatively lower degree of *O*-sulfation was named as K5-OS_1. *N*-deacetylated/*N*-sulfated K5 polysaccharide (K5-NS) was synthesized with the *N*-deacetylation step in 2 M NaOH, and then with a further step of *N*-sulfation. K5-NS was run through a cation exchange column (IR-120 H^+; Bio-Rad) at 10 °C and was neutralized with 15% tetrabutylammonium hydroxide in water. After freeze drying, the sample was dissolved in *N*,*N*-dimethylformamide, and pyridine–sulfotrioxyde complex was added to obtain *N*,*O*-sulfated K5 polysaccharide (K5-NS,OS).

2.5. Characterization of K5 derivatives

NMR spectra were recorded in D_2O (99.9%, Sigma-Aldrich) with a Bruker Advance 400 MHz spectrometer. D_2O at 4.75 ppm was used as a reference line. Molecular weight determination was performed by gel permeation chromatography with a multi-angle light scattering detector (GPC–MALS) analysis. Percentages of carbon and sulfur were determined by elemental analysis. The degree of sulfation (DS) was defined as the average number of sulfate ester groups inserted in each disaccharide unit, which was calculated in a similar way to that used by Melo et al. Disaccharide analysis was carried out using USP-based methods. Briefly, each substrate was treated with a mixture of heparinase I solution, heparinase II solution, and heparinase III solution, and incubated for 48 h at 30 °C. Strong anion exchange (SAX)-HPLC was performed to analyze the degradation products of K5 polysaccharide and sulfated K5 polysaccharide derivatives.

2.6. Macrophage activation by sulfated K5 polysaccharide derivatives in vitro

2.6.1. RAW264.7 proliferation assay

The cytotoxic effects of sulfated K5 derivatives on RAW264.7 cells were assessed using MTT assay. In brief, cells were seeded at 1×10^5 cells/mL in a 96-well plate for 24 h and then were cultured with different concentrations of sulfated K5 derivatives for 24 h or 48 h. After incubation, MTT was added to each well to a final concentration of 0.5 mg/mL. The plates were further incubated for 4 h, and then the produced formazan crystals were dissolved in DMSO. The absorbance at 570 nm was measured by microplate ELISA reader.

2.6.2. Determination of cytokine production

RAW264.7 cells were cultured for 24 h with the samples in different concentrations as above. Then supernatants were harvested

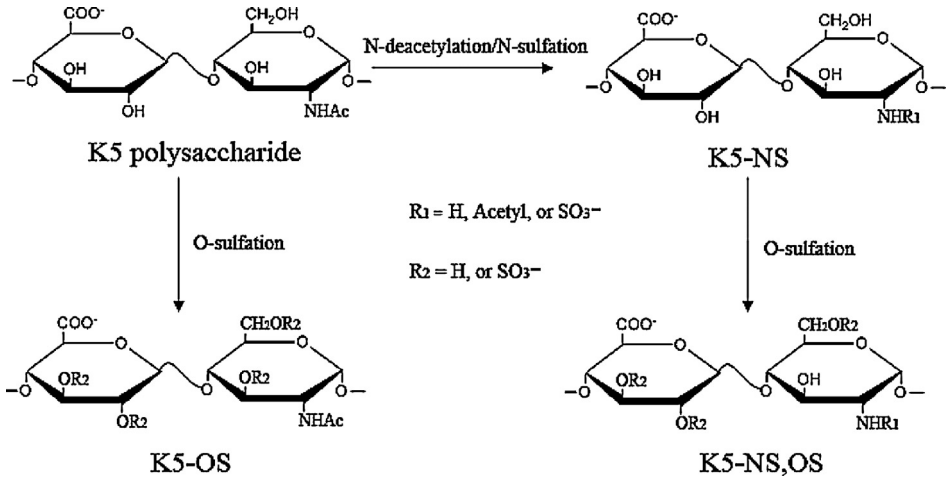

Fig. 1. Schematic illustration of the synthesis of sulfated derivatives from the K5 polysaccharide.

and the concentrations of TNF-α, IL-1β were determined using ELISA kits, according to the manufacturer's instructions. Standard curves were used for calculation of cytokine concentration.

2.7. Spleen lymphocytes activation by sulfated K5 polysaccharide derivatives in vitro

2.7.1. Preparation of spleen lymphocytes

The mice were sacrificed by cervical dislocation, followed by aseptic removal of the spleen with scissors and forceps. The spleen then was grinded and gently passed through a sterilized ion mesh (200 mesh) to obtain single spleen cell suspensions, as described previously (Ahn et al., 2011). After the red blood cells were removed by Tris–NH_4Cl solution, the remaining cells were washed twice with PBS and adjusted to a density of 1×10^7 cells/mL in RPMI 1640 complete medium containing 10% FBS.

2.7.2. Spleen lymphocytes proliferation assay

The assay of spleen lymphocytes proliferation was implemented according to the previous method (Yi et al., 2012). Briefly, splenocyte suspension was placed in a 96-well culture plate with or without ConA (10 μg/mL), LPS (10 μg/mL). Con A and LPS were positive controls for the proliferation of T and B cells, respectively. The filter-sterilized samples in different concentrations as above were added into the cell wells. After incubation for 48 h at 37 °C in a humidified 5% CO_2 incubator, cell proliferation was estimated based on the method of the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) according to the manufacturer's manual.

2.7.3. Cytokines and immunoglobulins analysis in cultures of spleen lymphocytes

The effects of sulfated K5 derivatives on the production of IL-2, IFN-γ, IgG1a, and IgG2b were established via ELISA. Briefly, the lymphocytes were incubated with different derivatives, ConA (10 μg/mL) or LPS (10 μg/mL) for 48 h. The supernatants were collected to measure IL-2, IFN-γ, IgG1a, and IgG2b expression levels. The cytokines and immunoglobulins expression levels were calculated based on the linear portion of the standard curve.

2.7.4. Natural killer (NK) cell cytotoxicity assay

Splenocytes was prepared as the effector cell for splenic NK cell activity assay according to previous report (Yi et al., 2011). In brief, the same volume of YAC-1 cells (1×10^5 cells/mL) and splenocytes (1×10^6 cells/mL) were added to the 96 well plate (effector cells:target cells = 10:1). The plate was incubated for 6 h at 37 °C in a humid atmosphere with 5% CO_2, followed by another 4 h with MTT. Then, the produced formazan crystals were dissolved in DMSO and the absorbance was measured at 570 nm. The percentage of NK cell cytotoxicity was calculated from the following formula: NK cell cytotoxicity (%) = $[OD_T - (OD_S - OD_E)]/OD_T \times 100$, where OD_S, OD_E and OD_T represented the OD values of stimulated group, effector control and target control, respectively.

2.8. Statistical analysis

The results were presented as mean ± standard deviation. One-way analysis of variance (ANOVA) was used for statistical analysis. The Tukey method for the multiple comparisons among the groups was used to determine the significant differences.

3. Results

3.1. Characterization of sulfated K5PSs

The sulfated K5PS compounds were synthesized as described in the Materials and Methods section. ^{13}C NMR spectroscopy was used to characterize the substitution patterns of sulfate group in K5 and its sulfated derivatives. The ^{13}C NMR spectra of K5PS (Fig. 2) are similar to the previously published spectra for heparosan (Wang et al., 2010b ; Zhang et al., 2012). By comparing the ^{13}C NMR spectra of unsubstituted K5PS and K5-OS_2 (Fig. 2), a low-field shift of the C-6 signal of the primary CH_2-group in the *N*-acetylglucosamine (GlcNAc) unit from 59.35 ppm to 68.95 ppm was observed after sulfation. A nearly complete sulfation of the C-6 position was assumed, even in the case of the low-sulfated derivative K5-OS_1 (data not shown here). As shown in Fig. 2, sulfation can be speculated to also occur to a lesser extent on the remaining secondary OH groups of the GlcNAc (C-3) and the glucuronic acid (GlcA) unit (C-2′, C-3′) in the high-sulfated derivative K5-OS_2. The chemical shift (21.91 ppm) of methyl carbon of the GlcNAc in K5-NS and K5-NS,OS disappeared, which indicated that K5-NS and K5-NS,OS were fully *N*-deacetylated (Fig. 2). Furthermore, the C-2 signal of the GlcNAc exhibited a chemical shift from 53.22 ppm to 57.61 ppm after *N*-deacetylation/*N*-sulfation.

Table 1a presents the data on the molecular weight (*Mw*) of K5 and its sulfated derivatives. The K5 polysaccharide isolated from *E. coli* K5 capsule had a weight average molecular weight of 72.2 kDa, which was similar to that described in the literature

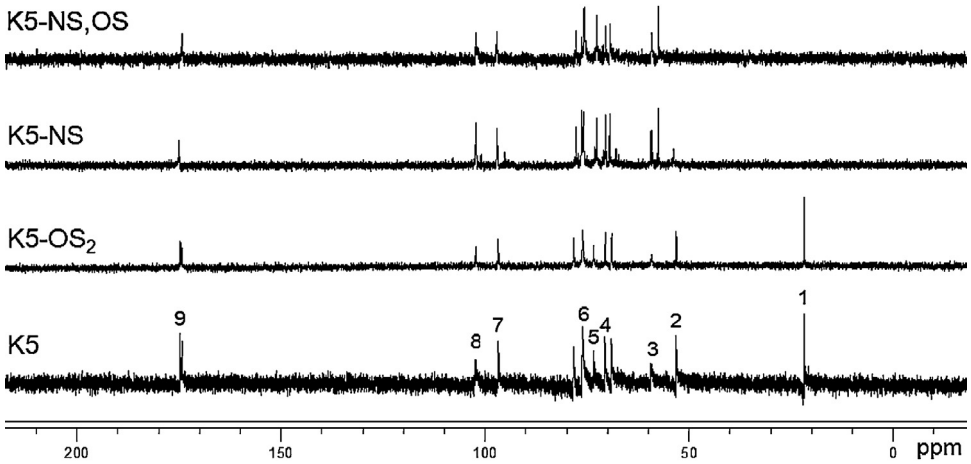

Fig. 2. ^{13}C NMR spectra of K5, K5-OS_2, K5-NS, and K5-NS,OS. The carbon signals are assigned as follows: (1) methyl C of the GlcNAc, 21.91 ppm; (2) C-2 atom of the GlcNAc, 53.22 ppm; (3) C-6 atom of the GlcNAc, 59.35 ppm; (4) C-3 atom of the GlcNAc, 70.65 ppm; (5) C-2 atom of the GlcA, 73.46 ppm; (6) C-3 atom of the GlcA, 75.97 ppm; (7) C-1 atom of the GlcNAc, 96.78 ppm; (8) C-1 atom of the GlcA, 102.30 ppm; and (9) C atom of the carboxyl group of the GlcA, 174.71 ppm.

Table 1a
Number average molecular weight (*Mn*), weight average molecular weight (*Mw*), polydispersity index (PDI), and elemental analysis of K5 and its sulfated derivatives.

Sample	GPC-MALS			Elements (% w/w)		
	Mn (kDa)	*Mw* (kDa)	PDI	Carbon	Sulfur	DS[a]
K5	58.5	72.2	1.2	36.51	0	0
K5-OS_1	52.8	65.8	1.3	35.32	0.84	0.10
K5-OS_2	54.9	68.9	1.3	31.16	2.26	0.33
K5-NS	6.9	9.3	1.4	29.86	4.41	0.66
K5-NS,OS	7.7	10.2	1.3	26.01	5.99	1.04

[a] DS, the degree of sulfation. DS = 4.5 × (S%/C%).

Table 1b
Summary of the disaccharide compositions of sulfated derivatives.

Disaccharide composition	K5	K5-OS_1	K5-OS_2	K5-NS	K5-NS,OS
ΔUA-GlcNAc (%)	99.4	84.5	64.6	2.3	2.2
ΔUA-GlcNS (%)	0	0	0	95.4	76.8
ΔUA-GlcNAc6S (%)	0	10.1	19.5	0	0.1
ΔUA2S-GlcNAc6S (%)	0	0	10.3	0	0
ΔUA-GlcNS6S (%)	0	0	0	0	12.9
ΔUA2S-GlcNS6S (%)	0	0	0	0	0.6

(Wang et al., 2010b) The *Mw* of K5 sulfated derivatives was much lower than that of K5 polysaccharide, which was possibly due to the hydrolysis of the glycosidic bond during the sulfation process (Yang et al., 2013). To determine the degrees of sulfation in each of the sulfated compounds, we performed elemental analysis. Sulfated K5PS species with different DS were obtained as summarized in Table 1a. Synthesized polysaccharides were digested with a mixture of heparinase solutions, and the resulting disaccharides were analyzed using SAX-HPLC. The disaccharide components of different compounds are shown in Table 1b. The analysis of K5-NS showed a disaccharide component of the structure of ΔUA-GlcNS, and small amounts of ΔUA-GlcNAc. The modification by *N*-deacetylation/*N*-sulfation elevated the levels of ΔUA-GlcNS and ΔUA-GlcNS6S, confirming the structure of K5-NS,OS. Also, *O*-sulfation resulted in a predominant 6-*O*-sulfation of *N*-acetylglucosamine residues in all the *O*-sulfated derivatives. Thus, the analysis of K5-OS_1 afforded an increase in the level of the disaccharide ΔUA-GlcNAc6S compared with that of K5, whereas K5-OS_2 was more heterogeneous in *O*-positions, with 20% of its sequence being represented by ΔUA-GlcNAc6S disaccharide units, and 10% of the disaccharide ΔUA2S-GlcNAc6S.

3.2. Effects of sulfated K5PSs on growth of RAW 264.7 cells

To determine whether the HS mimetics from K5 capsular polysaccharide is cytotoxic to RAW 264.7 cells, we analyzed the cell viability at various doses of sulfated K5PSs (1, 10, 50, and 100 μg/mL) via MTT assay. The doses used did not show any cytotoxic effect on RAW 264.7 cells (Fig. 3). When RAW 264.7 cells were stimulated with the samples (100 μg/mL), the cell viability was significantly higher than that of the control group ($p < 0.05$). We also observed a greater positive effect (22.6 ± 4.7% or 28.3 ± 4.0%) on cell proliferation when K5-OS_2 was added at a concentration of 50 or 100 μg/mL. No significant changes were found in the viability of cells treated with other samples compared with the control.

3.3. Effects of sulfated K5PSs on macrophage TNF-α and IL-1β production

The results on cytokine production of four different polysaccharides are shown in Fig. 4. K5-OS_2 (1 μg/mL) demonstrated higher TNF-α and IL-1β induction than the control at low concentrations, and this induction increased with increasing concentrations. TNF-α and IL-1β productions in the macrophages treated with 100 μg/mL of K5-OS_2 markedly increased up to 1180.4 ± 34.3 pg/mL (Fig. 4A) and 123.1 ± 0.1 pg/mL (Fig. 4B), respectively, whereas the levels of TNF-α and IL-1β were 871.0 ± 13.3 pg/mL and 96.1 ± 2.0 pg/mL, respectively, in the cells treated with the control ($p < 0.05$). At 100 μg/mL concentration, the IL-1β level treated with K5-OS_1 was 114.1 ± 0.4 pg/mL ($p < 0.05$), and TNF-α was induced at low levels (935.3 ± 26.7 pg/mL) compared with that in the normal control ($p > 0.05$). By contrast, K5-NS at concentrations of 1, 10, 50, and 100 μg/mL exhibited difficulty in significantly increasing the production of TNF-α and IL-1β in RAW 264.7 cells when stimulated for 24 h. K5-NS,OS increased IL-1β release from macrophages in a dose-dependent manner, and the production reached 120.5 ± 3.5 pg/mL at 100 μg/mL concentration. Notably, K5-NS,OS-activated RAW 264.7 cells produced considerably higher concentrations of TNF-α than the LPS-treated control (933.4 ± 18.9 pg/mL), with levels reaching approximately 1161.8 ± 113.4 pg/mL at 100 μg/mL K5-NS,OS concentration ($p < 0.05$).

3.4. Effects of sulfated K5PSs on splenic lymphocyte proliferation

By performing the CCK-8 assay, we established the viability of splenic lymphocytes in the presence of sulfated K5PSs after 48 h incubation. Fig. 5 shows that during single stimulation, the proliferation rate of K5-OS_2 group was extremely significantly higher

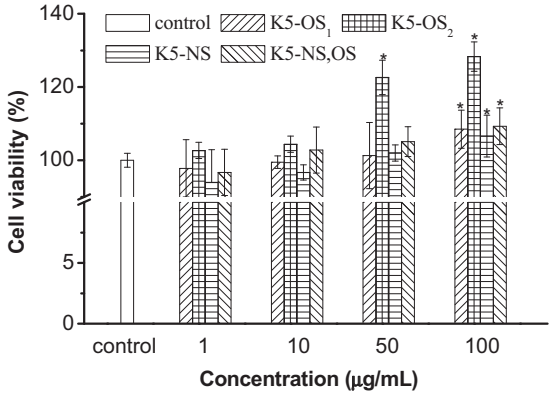

Fig. 3. Cell proliferation analysis with different concentrations of sulfated K5PSs in comparison to the control. The percentage of untreated cell viability was calculated as 100%. The result represented the mean ± SD ($n = 3$). * $p < 0.05$ compared with the control group.

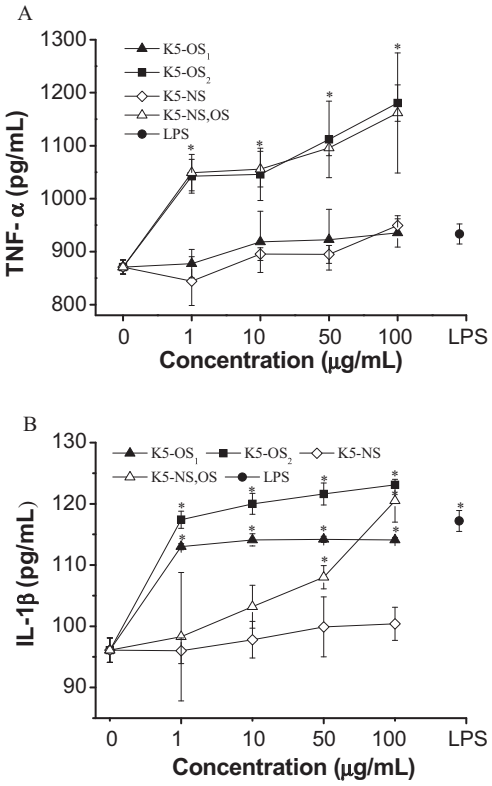

Fig. 4. Effect of sulfated K5PSs on cytokine production. TNF-α (A) and IL-1β (B) in RAW264.7 cells were detected using ELISA kits for 24 h in the presence or absence of 1, 10, 50, or 100 μg/mL sulfated K5PSs. LPS (10 μg/mL) was used as a positive control to activate RAW264.7 cells. Values represent mean ± SD and $n = 3$. The p values are shown as $p < 0.05$ *vs.* the negative control group (untreated group). A value of $p < 0.05$ denoted the presence of statistically significant difference according to the Tukey method.

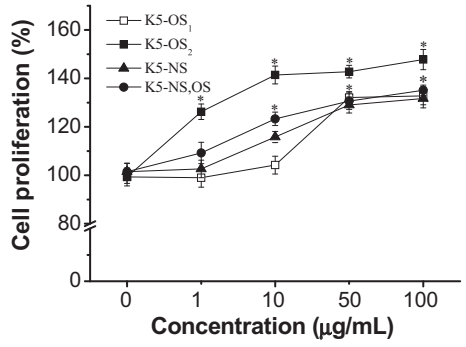

Fig. 5. Stimulation effects of sulfated K5PSs on spleen lymphocyte proliferation *in vitro*. The percentage of untreated cell proliferation was calculated to be 100%. * $p < 0.05$ indicated significant difference *vs.* the untreated control group.

than that of the untreated group at all test concentrations (1, 10, 50, and 100 μg/mL) ($p < 0.05$), with the highest proliferation rate of 147.8 ± 5.2% compared with the control (100%). A remarkable activity from K5-NS,OS was observed at concentrations higher than 10 μg/mL compared with the control group ($p < 0.05$), with 32.8% increase in lymphocyte proliferation activity at 100 μg/mL concentration. When the same dose was used, the proliferations obtained with K5-OS_1 and K5-NS were almost the same ($p > 0.05$), resulting in the range from 102.6 ± 9.6% to 131.7 ± 10.9%.

ConA stimulated the proliferation of lymphocytes with 31.2% increase compared with the untreated group. Furthermore, the coexistence of K5-OS and ConA significantly increased lymphocyte proliferation (except 1 μg/mL of K5-OS_1) compared with the ConA group ($p < 0.05$) (Table 2). The cell proliferation of lymphocytes incubated with K5-OS_2 and ConA ranged from 159.0 ± 14.1% to 215.2 ± 9.0% (the ConA group as 100%). Table 2 shows that K5-OS_2 stimulated LPS-induced spleen lymphocyte proliferation in the dose range of 10 μg/mL to 100 μg/mL, and the cell proliferation ranged from 121.2 ± 19.7% to 148.0 ± 18.8% (the LPS group as 100%). LPS individually enhanced the proliferation activity of lymphocytes by 21.2% compared with the negative group (untreated) during single stimulation. In addition, K5-NS or K5-NS,OS did not exhibit stimulating activities for lymphocytes co-incubated with ConA or LPS at the tested concentration (except 100 μg/mL of K5-NS,OS) compared with control cultures only containing ConA or LPS.

Table 2
Effects of sulfated K5PSs on ConA- or LPS-induced spleen lymphocyte proliferation *in vitro*.

Group	Concentration (μg/mL)	Proliferation rate (%)	
		ConA	LPS
Control (ConA or LPS)	10	100.0 ± 5.4	100.0 ± 11.3
K5-OS_1	1	101.9 ± 5.4	87.0 ± 6.8
	10	121.1 ± 0.9[a]	116.4 ± 15.8
	50	142.0 ± 14.2[a]	119.0 ± 15.1[b]
	100	187.6 ± 18.6[a]	124.5 ± 13.5[b]
K5-OS_2	1	159.0 ± 14.1[a]	106.3 ± 11.6
	10	159.4 ± 21.4[a]	121.2 ± 19.7[b]
	50	171.1 ± 16.6[a]	133.7 ± 9.0[b]
	100	215.2 ± 9.0[a]	148.0 ± 18.8[b]
K5-NS	1	99.9 ± 1.8	82.5 ± 9.9
	10	100.7 ± 1.2	101.1 ± 12.4
	50	107.4 ± 10.9	105.9 ± 15.5
	100	109.3 ± 20.6	108.6 ± 9.8
K5-NS,OS	1	100.2 ± 5.2	96.0 ± 11.7
	10	106.2 ± 2.2	106.1 ± 13.9
	50	109.6 ± 7.3	112.5 ± 3.5
	100	130.7 ± 8.6[a]	129.8 ± 10.3[b]

Data were expressed as mean ± SD of three independent experiments. The percentage of ConA- or LPS-induced cell proliferation was taken as 100%. Different letters in the figure represent statistical difference at $p < 0.05$.
[a] $p < 0.05$ significantly different from the ConA control.
[b] $p < 0.05$ significantly different from the LPS control.

3.5. Effects of sulfated K5PSs on expression levels of IL-2, IFN-γ, IgG1a, and IgG2b in lymphocytes

Cytokine IL-2 or IFN-γ possesses a key function in the activation of T lymphocytes (Wang et al., 2013a). The results showed that sulfated K5PS treatment elevated the expression of IL-2 and IFN-γ (Fig. 6A and B). As positive control, Con A (10 μg/mL) could significantly induce IL-2 and IFN-γ production. K5-OS_1, K5-OS_2, and K5-NS,OS increased the expression levels of IL-2 and IFN-γ in a dose-dependent manner, and the cytokine expression levels were significantly increased in high concentration (* $p < 0.05$). The effects were visible at 50 or 100 μg/mL of K5-OS_2 for IL-2 production and 100 μg/mL of K5-OS_2 for IFN-γ production compared with the positive control (# $p < 0.05$). By contrast, K5-NS had no influence on IL-2 and IFN-γ.

To identify the effects of sulfated K5PSs on B cell function, ELISA was carried out to measure IgG1a and IgG2b productions in lymphocytes after sulfated K5PS treatment. As shown in Fig. 6C and D, the lymphocytes treated with either 50 or 100 μg/mL of K5-OS_1 or K5-OS_2 produced slightly more IgG1a and IgG2b, which were secreted by B cells after activation (Ahn et al., 2013). However, K5-NS and K5-NS,OS did not affect the IgG expression of B lymphocytes compared with the normal control ($p > 0.05$). LPS (10 μg/mL) enhanced the secretion and production of IgG1a and IgG2b compared with the normal group (* $p < 0.05$). Interestingly, K5-OS_2 at 100 μg/mL concentration had a significant stimulatory

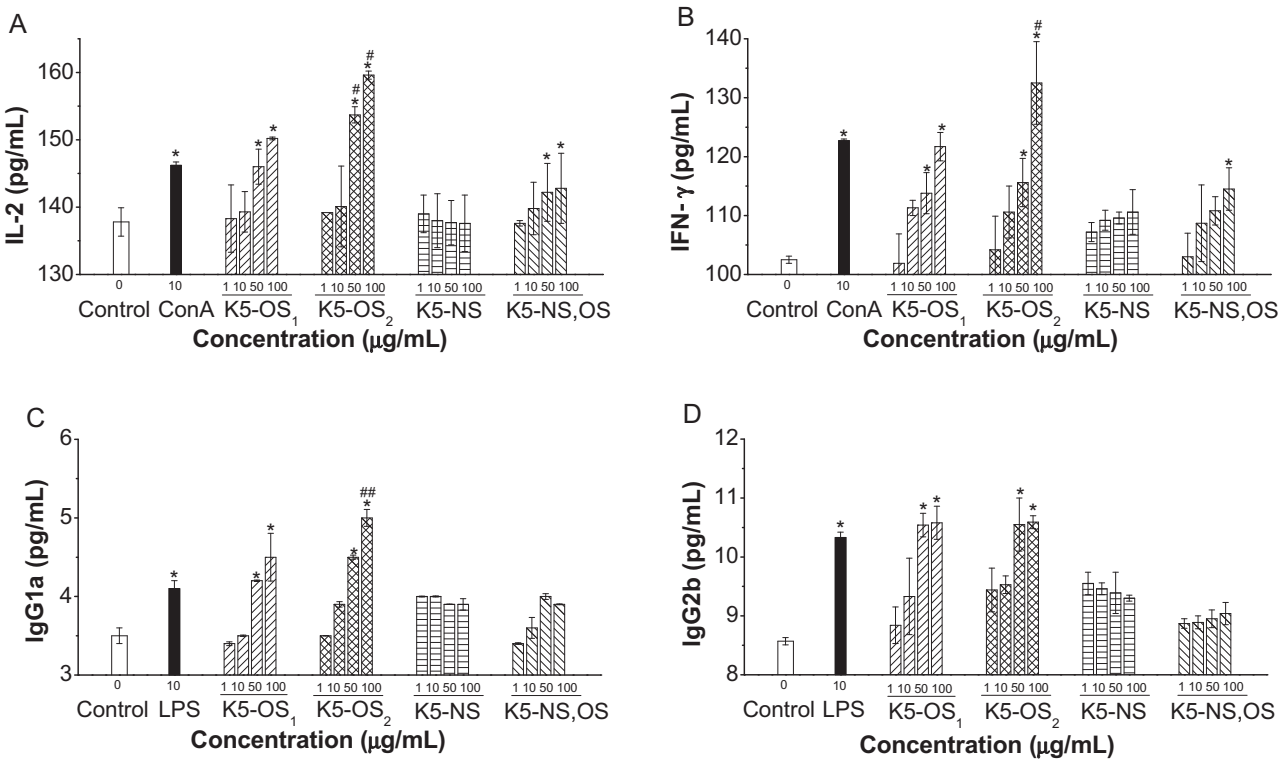

Fig. 6. Effects of sulfated K5PSs on the expressions of IL-2, IFN-γ, IgG1a, and IgG2b. The expression levels of IL-2 (A), IFN-γ (B), IgG1a (C), and IgG2b (D) were assessed through ELISA assay. Values are mean ± SD of triplicate determinations. * $p < 0.05$ compared with the RPMI-1640 control, $^{\#}$ $p < 0.05$ compared with the ConA control, and $^{\#\#}$ $p < 0.05$ compared with the LPS control.

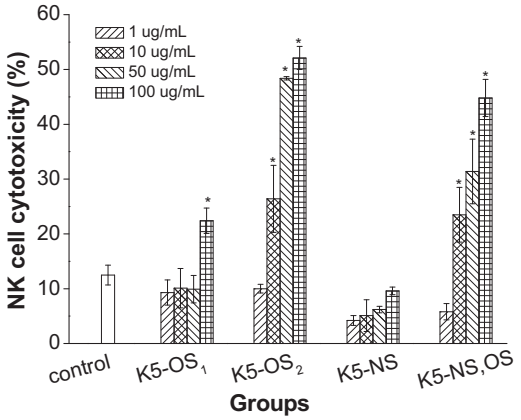

Fig. 7. Effects of sulfated K5PSs on the cytotoxicity of NK cells. NK cell cytotoxicity was assessed through MTT assay and expressed as means ± SD ($n = 3$). The statistically significant differences among the groups were evaluated using ANOVA followed by Tukey test. Asterisk (*) in the figure represents statistical difference at $p < 0.05$.

effect on inducing the release of IgG1a compared with the level in LPS-treated cells ($^{\#\#}$ $p < 0.05$).

3.6. Effects of sulfated K5PSs on NK cell cytotoxicity

We measured the cytotoxic activity of splenocytes against NK cell-sensitive YAC-1 cells via MTT. Fig. 7 shows that all the compounds except K5-NS enhanced NK cell cytotoxicity against YAC-1 cell at an adaptive dose compared with the control. The NK cell cytotoxicity of K5-OS_1 was significantly enhanced at the highest dose (100 μg/mL) compared with the control group ($p < 0.05$). K5-OS_2 and K5-NS,OS promoted NK cell cytotoxicity in the dose range of 10 μg/mL to 100 μg/mL in a dose-dependent manner ($p < 0.05$), and a significant difference was found between the two samples at the same concentration of 50 or 100 μg/mL ($p < 0.05$). The NK cell cytotoxicity ranged from 26.4 ± 6.1% to 52.1 ± 2.1% at 10 μg/mL to 100 μg/mL concentration of K5-OS_2. This result exhibits that K5-OS_2 has the best capability to improve the cytotoxicity of NK cells.

4. Discussion

It is well known that macrophages are one kind of multipotential cells that participate in both specific and non-specific immune reactions (Lee et al., 2013; Park et al., 2013). In this study, the finding confirms that sulfated K5PSs induced macrophage activation without cytotoxicity in RAW 264.7 cells. It also indicated that sulfated polysaccharides at suitable concentrations significantly stimulated macrophage proliferation, respectively, and the action of K5-OS_2 was the strongest.

Moreover, cytokines are signaling molecules that control homeostasis of the organism by regulating cell differentiation, proliferation, and apoptosis, as well as defense functions, such as immune responses (Zhang & Dai, 2011). TNF-α produced by activated macrophages, T lymphocytes, and NK cells has a wide variety of effects and an important function as a key cytokine in immune and inflammatory reactions (Kouakou et al., 2013; Zhao et al., 2013). IL-1β is an important cytokine for regulation of immune responses, which is involved in a variety of cellular activities, including cell proliferation of T and B lymphocytes (Schepetkin et al., 2013). Based on the results, K5-OS_2 and K5-NS,OS remarkably stimulated RAW 264.7 cells to release TNF-α and IL-1β, which have been implicated as key mediators in the response to immunological stimuli.

Lymphocyte proliferation is an important index that evaluates cellular and humoral immune function. The results of the

experiment *in vitro* showed that K5-NS and K5-OS_1 possessed certain stimulative effects on lymphocyte proliferation, and the activity of K5-OS_2 was significantly higher. Proliferation of spleen lymphocyte induced by ConA *in vitro* may be used as a method to evaluate T lymphocyte activity, whereas that induced by LPS may be used to examine B lymphocyte activity (Wang et al., 2010a). During synergistical stimulation with ConA, the proliferation rate induced by K5-OS_2 group was highest, and at its minimum effective concentration the cell viability was significantly larger than that of the ConA control group. The proliferation rates induced by K5-OS_1 and K5-NS,OS at some concentrations were significantly larger than that of the ConA control group. K5-OS_2 exhibited strong induction effect on T cell proliferation because of ConA action. During synergistical stimulation with LPS, similar results were obtained; the proliferation assay showed that K5-OS_2 significantly promoted LPS-stimulated splenocyte proliferation *in vitro*. The present results indicated that K5-OS_2 promoted both murine T- and B-cell proliferation induced by mitogen, strengthened immunological response, and improved immunity. However, the potential mechanism involved in the promotion of LPS and ConA-induced proliferation was not clearly elucidated for K5-OS_2.

K5-OS_2 and K5-OS_1 not only enhance the proliferation of immune cells, but also stimulate the secretion of IL-2, IFN-γ, IgG1a, and IgG2b in lymphocytes. IL-2 is a type of cytokine signaling molecule in the immune system, which is normally produced during an immune response. It is also necessary for the proliferation, activation, and function of T lymphocytes (Feng et al., 2013; Zhang et al., 2011). IFN-γ, which is secreted by activated Th1-type cells, has a critical function in immunomodulatory effects (Wang, Zhu, & Lin, 2012). The Th1 cells not only secrete cytokines, but also stimulate the expression of IgG2a, IgG2b, and IgG3, which are B-lymphocyte productions in the immune system (Feng et al., 2013). Taken together, our data showed that K5-OS_2 and K5-OS_1 could enhance both cellular and humoral immunities. By contrast, our results indicate that K5-NS had no effect on the growth and activation of T and B lymphocytes. This result may be due to the important role of heparan sulfate with *O*-sulfated groups in the activation of immune cells, but we were not able to precisely correlate the exact structure with its immunomodulatory activity.

Similar results were obtained in the NK cell cytotoxicity assay. NK cells are part of the innate immune system and have a major function in protecting against infectious diseases and cancer. The special structures of sulfated K5PSs may differ from each other because of their different *Mw* and monosaccharide compositions, which resulted in their different effects on the cytotoxicity of NK cells. K5-OS_2 and K5-NS,OS upregulated the cytotoxicity of NK cells at 10 μg/mL to 100 μg/mL concentration. The highest doses where K5-OS_1 showed upregulation may be higher than 100 μg/mL. The NK cell cytotoxic regulation of K5-NS was not found.

The biological activity of a polysaccharide mainly depends on its molecular structure, *Mw*, degree of sulfation (Chandrasekaran et al., 2013; Zhang et al., 2014). The capsular K5 polysaccharide from *E. coli* has the same structure [→4) β-D-GlcA (1 → 4) α-D-GlcNAc (1→]$_n$ as the heparin precursor *N*-acetyl heparosan (Li et al., 2013). This study synthesized *N*-, *O*-, and *N*,*O*-sulfated K5 derivatives with different degrees of sulfation. K5-OS_1 had a low DS (0.10) in *O*-position, whereas K5-OS_2 had a relatively high DS (0.33). Numerous data have indicated that both DS and charge distribution modulate the biological activity of sulfated derivatives (Wang et al., 2013b). Comparing polysaccharides with similar *Mw*, our experimental results indicated that the immunomodulatory activity exhibited by K5-OS_2 with relatively high DS exceeded that K5-OS_1 with low DS, whereas K5-NS,OS exerted stronger stimulation on immunomodulatory activity than K5-NS did. This finding was in keeping with other study that highly *O*- and *N*,*O*-sulfated K5 derivatives are equally effective on endothelial cells in most of the *in vitro* assays, whereas the effectivity of their low sulfated counterparts is poor (Borgenström et al., 2007). In contrast, heparin, a special haparan sulfate with about 2.5 sulfate groups per disaccharide, has a weak direct immunosuppressive action. The inhibitory effects of heparin on human lymphoproliferative response and NK cytotoxicity have been reported by previous studies (Górski, Wasik, Nowaczyk, & Korczak-Kowalska, 1991). Therefore, it is possible that there was an optimum degree of sulfation for heparan sulfate mimetics to reach the maximal immunomodulatory response. Previous studies have shown that *O*-sulfated K5 derivatives inhibit both expression and production of inflammatory cytokines by human mononuclear cells (Gori et al., 2004). The comparison between the structures and activities of four sulfated K5PSs indicated that sulfate substitution position was a key structural factor that determined the efficacy of stimulation of macrophages and splenic lymphocytes *in vitro*. The sulfation in *O*-position of K5 polysaccharide appeared to be more effective than the sulfate in *N*-position. Our results suggested that the higher the DS in *O*-position of K5 polysaccharide, the stronger the activity of macrophages and lymphocytes would be. In addition, according to previous reports, the immunomodulatory activity of the sulfated polysaccharide depended on its molecular weight. In this study, K5-NS or K5-NS,OS with lower *Mw* showed the lower activity than K5-OS2 with higher *Mw*, which indicated that biological activity correlated with increased molecular weight of polysaccharides, as we found previously in a number of studies (Kouakou et al., 2013). It seems that the activity of heparan sulfate mimetics is not a function of one single factor but rather of a combination of factors. Taken together, further studies should clarify the detailed mechanisms involved in the immune enhancement-like properties of K5-OS_2 to support the present findings. Many studies have confirmed that chemically sulfated glycosaminoglycans showed toxicity in both animals and humans (Li et al., 2009). It is more important to us to demonstrate the immunodulatory activity of sulfated K5 polysaccharides *in vivo*.

In conclusion, among the four sulfated K5PSs, K5-OS_2 strongly modulated the immune response by stimulating TNF-α and IL-1β in the mouse macrophage RAW 264.7 cell line. K5-OS_2 increased splenocyte proliferation and enhanced both murine T- and B-cell proliferation and activation. High sulfation in *O*-position of K5 polysaccharide may be required for their immunomodulatory activities. We conclude that K5-OS_2 may be useful as an immune stimulant and a promising agent with immune-stimulating effects, which may also be indirectly involved in antitumor activity through improving immunologic function.

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