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The effects of chitosan oligosaccharide on the activation of murine spleen CD11c$^+$ dendritic cells via Toll-like receptor 4

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ABSTRACT

To investigate the effects of chitosan oligosaccharide (COS) on dendritic cells (DCs) and the role of Toll-like receptor 4 (TLR4) in this immune process, Murine spleen CD11c$^+$ dendritic cells (SDCs) were isolated and cultured with S-COS and B-COS with polymeration degree of 3–7 and 7–16, respectively. The results showed that B-COS up-regulated the expressions of MHCII and CD86 on SDCs, promoted the secretion of TNF-α from SDCs. SDCs treated with B-COS stimulated the proliferation of the CD4$^+$T cells. However, these effects were not observed on SDCs treated with S-COS. Importantly, silencing the TLR4 expressions on SDCs by RNA interference approach attenuated the expression of CD86, MHCII on SDCs, and the secretion of TNF-α from SDCs, and the stimulating CD4$^+$T cells proliferation capacity of SDCs induced by B-COS. These results suggest that B-COS, but not S-COS, promotes the activation of SDCs and TLR4 plays a bridge role in this process.

1. Introduction

Chitin, a homopolymer of β-1,4 linked N-acetylglucosamine, is the second most abundant polysaccharides. Chitosan is the deacetylated derivative of chitin, and previous works revealed that chitosan plays a role in immune responses for plant and animal cells (Porporatto, Bianco, & Correa, 2005; Villiers et al., 2009). Chitosan oligosaccharide (Chitooligosaccharides, COS), derived from chitosan by enzymatic hydrolysis (Zhang, Du, Yu, Mitsutomi, & Aiba, 1999), is a new kind of biofunctional material which exhibits improved biological activities when compared with chitosan (Cho et al., 2008; Moon et al., 2007; Nam, Kim & Shon, 2007; Palma-Guerrero, Jansson, Salinas, & Lopez-Llorca, 2008; Rahman et al., 2008; Yoon, Moon, Park, Im, & Kim, 2007), such as inhibiting growth of bacteria and fungi, exerting anti-tumor activity, and acting as immunopotentiating effectors.

Dendritic cells (DCs) are central players in the process of the immune response. DCs are present in an immature state in peripheral tissues as sentinels to detect pathogens, influencing both innate and adaptive immunity immediately upon invasion. DCs are equipped with a range of pattern recognition receptors (PRRs), such as Toll-like receptor 4 (TLR4). Upon activation through PRRs, DCs turn mature and up-regulate the expressions of surface molecules that are essential for T cells activation, such as CD80, CD86 and MHCII, as well as the secretion of cytokines (Kadowaki et al., 2001; Michelsen et al., 2001; Qi, Denning & Soong, 2003). DCs activate T cells, resulting in specific acquired immunity. During T-cell activation, DCs also provide the T cells with signals which direct T cells to polarize and to develop into T helper1 (Th1) cells, T helper2 (Th2) cells, regulatory T cells (Treg) or IL-17 producing T helper cells (Th17). Thus, maturation of DCs is essential for the appropriate initiation of the subsequent adaptive immune response.

Like DCs, lymphocytes and macrophages are the antigen-presenting cells (APC) which perform as bridges in the immune response. The effects of COS on the activation of lymphocytes and macrophages have been reported by previous studies (Feng, Zhao & Yu, 2004; Maeda & Kimura, 2004). Most recently, it was reported that Chitosan activated DCs (Villiers et al., 2009). However, there is less report about the effect of COS on dendritic cells activation. The mechanism of COS activates DCs is not clearly addressed thus far. In the present study, the effects of COS with different polymeration degree on the SDCs activation and the role of TLR4 in this process are investigated.

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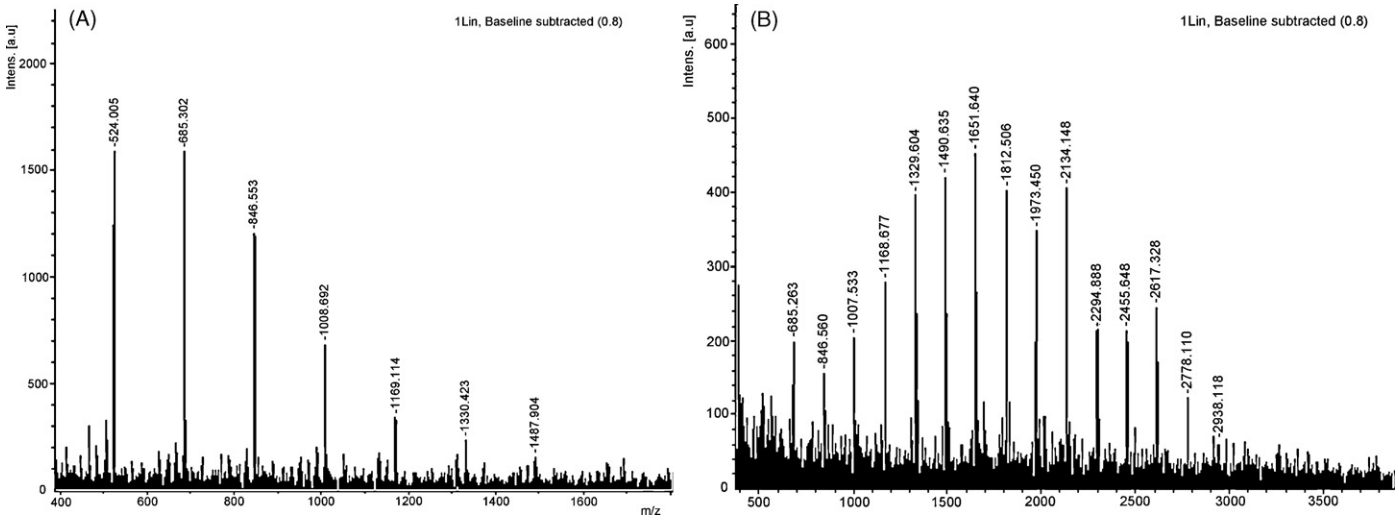

Fig. 1. Mass spectrogram of S-COS sample (A) and B-COS sample (B).

2. Materials and methods

2.1. Animals

Female BALB/c mice and wild-type C57BL/6 mice at age of 4–6 weeks were purchased from Dalian Medical University and allowed to acclimate to the animal facility for 1 week prior to any procedures. Mice were maintained on a 12 h light/dark schedule with the lights on at 6 a.m. Aggressive intruders were individually housed. All animal operations have been approved by Dalian Medical University Animal Ethic Committee.

2.2. Preparation of chitosan oligosaccharide (COS)

COS was prepared from enzymic hydrolysis chitosan according to our previous methods (Zhang et al., 1999). In brief, chitosan (5 g) was dissolved in 2% AcOH (100 ml), and then the pH of the solution was adjusted to 5.6. Enzyme mixture (5 mg) in 0.05 mol/L acetate buffer was added and the mixture was incubated for 30 min at 40 °C. The reaction was stopped by boiling for 10 min. The hydrolyzates were filtered on a hollow-fiber membrane. The pH of the COS solution was adjusted to 8 by slowly addition NH_4OH. The insoluble precipitate was filtered, washed and lyophilized, assigned as B-COS. The filtrate COS solution was lyophilized, assigned as S-COS. MALDI-TOF mass spectrometry analysis indicated that S-COS and B-COS are of polymeration degree of 3–7 and 7–16, respectively (Fig. 1).

2.3. Isolation of SDCs

SDCs were isolated from female BALB/c mice by using CD11c (N418) Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) (Pulendran et al., 1997) according to the manufacturer's instruction. Briefly, the spleens were gently teased and incubated with 2 mg/ml Collagenase D (Roche Diagnostics, Germany) for 30 min to obtain single cell suspension. The cells were harvested and washed with phosphate-buffered saline (PBS) containing 0.5% BSA and 2 mM EDTA, followed by incubation with mouse CD11c antibody-labeled microbeads at 4 °C for 30 min. Labeled cells were positively selected by magnetic separation using MS magnetic antigen cell separation (MACS). PE-CD11c antibody staining and Flow cytometry analysis indicated that the ratio of $CD11c^+$ SDCs in total cells isolated in this manner was up to 90 and 95% cells were alive. SDCs were adjusted to 1×10^6 cells/ml with complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin) and seeded into 12-well plates at 2 ml/well. The cells were incubated at 37 °C in 5% humidified CO_2.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the surface molecule expression of SDCs treated with B-COS and S-COS

SDCs were cultured with B-COS and S-COS at different concentrations (0, 40, 80, 160, 320 μg/ml), respectively for 24 h. Total RNAs were isolated from SDCs using Trizol (Invitrogen, USA). cDNA was synthesized using RT-PCR kit (TaKaRa, Japan) according to the manufacturer's instruction. The cDNA was amplified by PCR using the specific primers set for mouse TNF-α, IFN-γ, IL-10 and GAPDH as an internal control. Primers used were listed in Table 1. PCR analysis was performed under the following conditions: denaturation at 94 °C for 5 min, and then 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, extension for 30 s at 72 °C. The amplified products were analyzed by agarose gel electrophoresis, followed by ethidium bromide staining.

2.5. Enzyme-linked immunosorbent assay (ELISA) of the cytokine secretion of SDCs treated with B-COS and S-COS

SDCs were cultured with B-COS and S-COS at different concentrations (0, 40, 80, 160, 320 μg/ml), respectively for 24 h. The culture supernatants of SDCs were collected, centrifuged at 300 × *g* for 5 min and frozen at −20 °C. The contents of TNF-α, IFN-γ and IL-10 in culture supernatants were analyzed by using mouse cytokines ELISA kits (R&D Systems, USA).

Table 1
Primer sequences for RT-PCR.

Gene	Primer sequence
TNF-α	Forward: 5′-AGAAAGAAGCCGTGGGTTGG-3′ Reverse: 5′-CATGCCTAACTGCCCTTCCT-3′
IFN-γ	Forward: 5′-AGCGCTGACTGAACTCAGATTGTAG-3′ Reverse: 5′-GTCACAGTTTTCAGCTGTATAGGG-3′
IL-10	Forward: 5′-GGTTGCCAAGCCTTATCGGA-3′ Reverse: 5′-ACCTGCTCCACTGCCTTGCT-3′
GAPDH	Forward: 5′-GGCCGTGAAGTCGTCAGAAC-3′ Reverse: 5′-GCCACGATGCCCAGGAA-3′

Table 2
siRNA sequences.

siRNA	Target sequence
TLR4 SiRNA	Sense: 5′-CCCAAUUGACUUCAUUCAAGA-3′ Antisense: 5′-UUGAAUGAAGUCAAUUGGGUU-3′
Negative control	Sense: 5′-UUCUCCGAACGUGUCACGUTT-3′ Antisense: 5′-ACGUGACACGUUCGGAGAATT-3′

2.6. Silencing of TLR4 in SDCs with small interference RNA (siRNA)

To silence TLR4 expression, based on Aoki et al.'s paper, the siRNA specific murine TLR4 sequences [Genbank access number: NM_021297] were used. The target and scramble sequences were synthesized by the manufacturer (GenePharma, Shanghai, China) (Table 2). The siRNA was transfected into SDCs by Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. After transfected SDCs were incubated at 37 °C in 5% humidified CO_2 for 48 h, SDCs were harvested and washed twice in PBS containing 1% fetal calf serum (FCS) and 0.1% NaN_3. Then the cells were incubated for 30 min at 4 °C with PE anti-mouse TLR4 (Biolegend, USA) according to the manufacture's instruction, and three parallel samples of every group were set. After three washes with cold PBS, the cells were fixed in 1% polyformaldehyde and analyzed by flow cytometry (Becton Dickinson, USA). An isotype control was used for this antibody and gate was set using this isotype control. Data analysis was performed using the Cell Quest Software (Becton Dickinson, USA).

2.7. The phenotype assay by flow cytometry

SDCs with or without silencing TLR4 were treated by 160 μg/ml B-COS for 24 h. SDCs were harvested and washed twice in PBS containing 1% FCS and 0.1% NaN_3. Then the cells were incubated for 30 min at 4 °C with FITC anti-mouse CD86 and FITC anti-mouse MHCII (Biolegend, USA) respectively according to the manufacture's instruction, and three parallel samples of every surface molecule were set. After three washes with cold PBS, the cells were fixed in 1% polyformaldehyde and analyzed by flow cytometry (Becton Dickinson, USA). An isotype control was used for each antibody and gates were set using the isotype controls. Data analysis was performed using the Cell Quest Software (Becton Dickinson, USA).

2.8. Allogeneic mixed lymphocyte reaction (MLR)

Stimulatory capacity of SDCs was reflected in the primary allogeneic mixed lymphocyte reaction (MLR) (Sakurai, Yamada, Simamura, & Motoyoshi, 1996). Allogeneic T cells, as responder cells, were separated from spleen cell suspensions of wild-type C57BL/6 mice by a nylon wool column method (Lyons, 2000). T cells were labeled with 2.5 μmol/L carboxyfluorescein diacetate, succinimidyl ester (CFSE) following the instruction of the manufacturer (Invitrogen, USA). SDCs with or without silencing TLR4 were treated with B-COS (160 μg/ml) for 24 h as stimulator cells. SDCs and T cells were implanted at the ratio of 1:10 for 5 days at 37 °C in 5% humidified CO_2. Simultaneously, T cells were treated with B-COS (160 μg/ml) for 24 h, then cultured for 5 days at 37 °C in 5% humidified CO_2. $CD4^+$T-cell were stained with PE/Cy5 anti-mouse CD4 (Biolegend, USA). Cell division was analyzed by flow cytometry detecting CFSE fluorescence (Dixon & Misfeldt, 1994).

2.9. Statistical analysis

Statistical comparison of data was performed using software SPSS13.0. Each assay was done at least three times. The data were listed as the means ± standard deviation, and Student's *t*-test was used to determine the significance of the differences in multiple comparisons. A *p*-value of less than 0.05 was considered to be statistically significant.

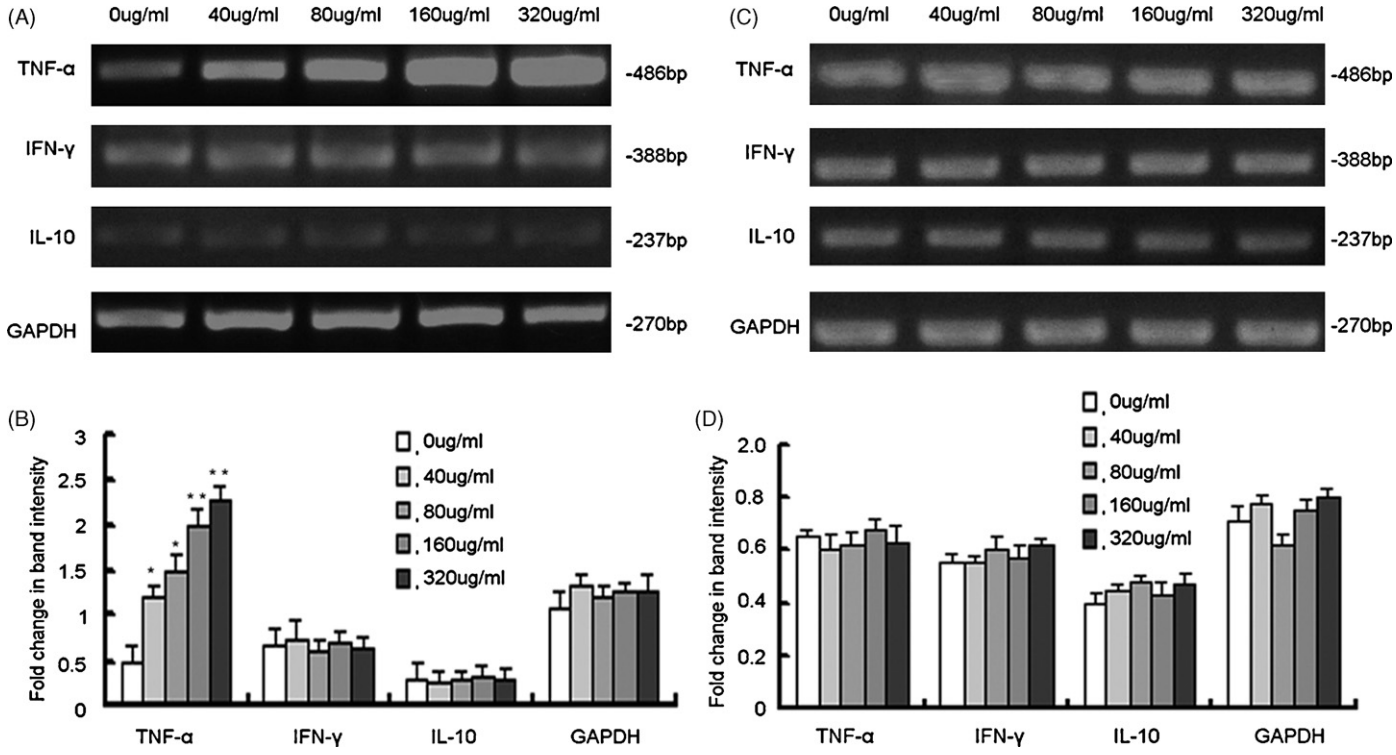

Fig. 2. Effects of B-COS and S-COS on TNF-α, IFN-γ and IL-10 expression in SDCs at mRNA levels. B-COS increased the TNF-α mRNA level in a dose-dependent manner, but not IFN-γ and IL-10 (A and B). No difference in the TNF-α, IFN-γ and IL-10 mRNA was observed in SDCs treated by S-COS (C and D).

3. Results

3.1. Effects of B-COS and S-COS on the cytokines mRNA levels in SDCs

SDCs were treated by B-COS and S-COS at different concentrations (0, 40, 80, 160, 320 μg/ml) for 24 h. The mRNA levels of TNF-α, IFN-γ and IL-10 were analyzed by RT-PCR. It was shown that B-COS, but not S-COS, increased the TNF-α mRNA level in a dose-dependent manner. There were no statistically significant difference among the effects of B-COS and S-COS on the SDCs at mRNA levels of IFN-γ and IL-10, respectively. These results suggest that B-COS may elicit SDCs to secrete TNF-α (Fig. 2).

3.2. Effects of B-COS and S-COS on the cytokine secretion of SDCs

SDCs were treated by B-COS and S-COS at different concentration (0, 40, 80, 160, 320 μg/ml) for 24 h, the protein amount of TNF-α, IFN-γ and IL-10 secreted were examined by ELISA. It was shown that B-COS (160, 320 μg/ml) significantly increased the TNF-α secretion in SDCs. However, no more TNF-α were observed in SDCs treated by S-COS. In regard to IFN-γ and IL-10, no more secretion induced by both B-COS and S-COS were detected, respectively. These findings are well consistent with the RT-PCR results (Fig. 3).

3.3. RNAi down-regulated the TLR4 expression of SDCs

After SDCs were transfected by TLR4 siRNA (scrambled siRNA as negative control) for 48 h, TLR4 expression levels were examined by flow cytometry (Fig. 4A). Compared with untransfected SDCs and the negative control, TLR4 expression was significantly decreased in the TLR4 siRNA-transfected SDCs. The results demonstrated that the TLR4 expression level was successfully down-regulated by TLR4 siRNA.

3.4. The secretion of TNF-α decreased in SDCs with silencing TLR4 treated by B-COS

After SDCs with silencing TLR4 were treated by B-COS (160 μg/ml) for 24 h, the supernatants were collected and cytokines protein levels were determined by ELISA. The results showed that B-COS increased the production of TNF-α in SDCs. Contrastly, along with silencing TLR4 expression, this effect was significantly reduced in SDCs (Fig. 4B).

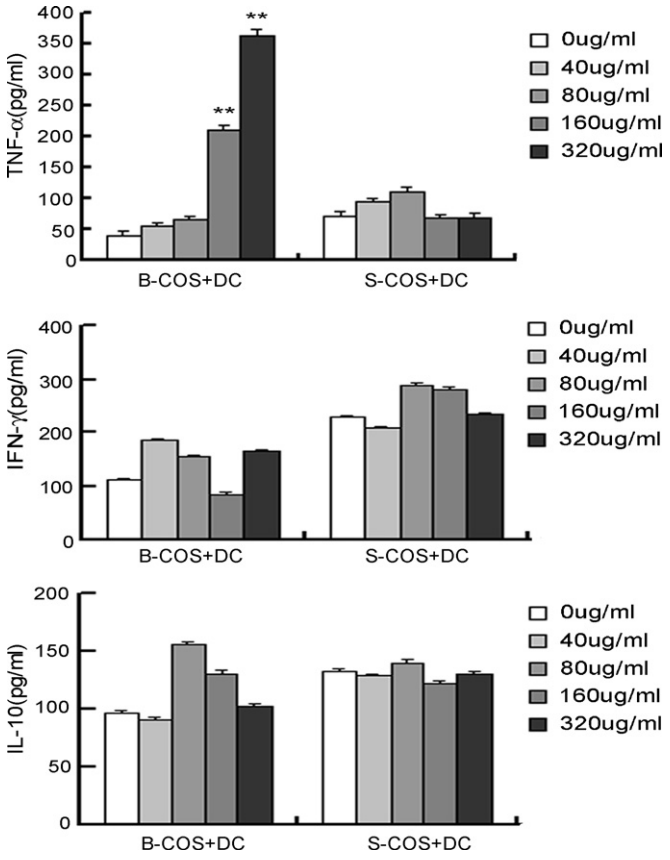

Fig. 3. Effects of B-COS and S-COS on the secretion of TNF-α, IFN-γ and IL-10 in SDCs. TNF-α secretion were increased significantly in SDCs streated by B-COS (160, 320 μg/ml), but not IFN-γ and IL-10. However, no more TNF-α, IFN-γ and IL-10 were observed in SDCs which were treated by S-COS.

3.5. The expressions of CD86 and MHCII reduced in SDCs with silencing TLR4 treated by B-COS

The changes of the phenotype, such CD86 and MHCII expressions, are hallmarks of DCs maturation. To investigate the role of TLR4 in the B-COS induced SDCs activation, SDCs with or without TLR4 siRNA transfection were cultured with 160 μg/ml B-COS for 24 h. Flow cytometry analysis results indicated that CD86 and MHCII expressions were significantly decreased in SDCs with

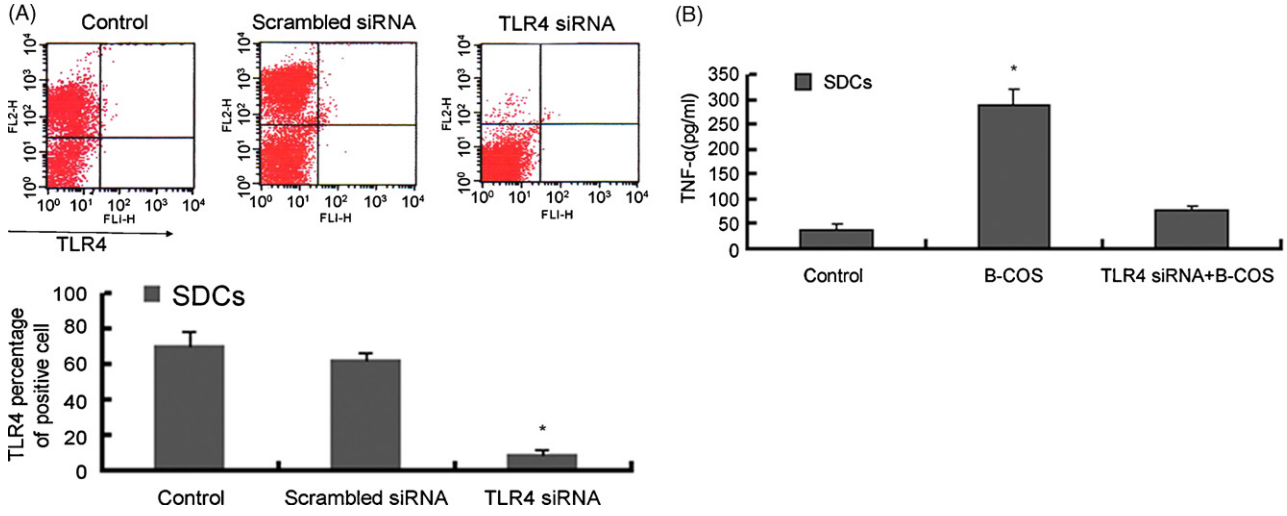

Fig. 4. TLR4 siRNA down-regulated TLR4 expression in SDCs. TLR4 expressions were not significantly changed in both nontransfected SDCs and the negative control. However, TLR4 expression was significantly decreased in the TLR4 siRNA-transfected SDCs (4A). TNF-α secretion decreased in SDCs with silencing TLR4 treated by B-COS (4B).

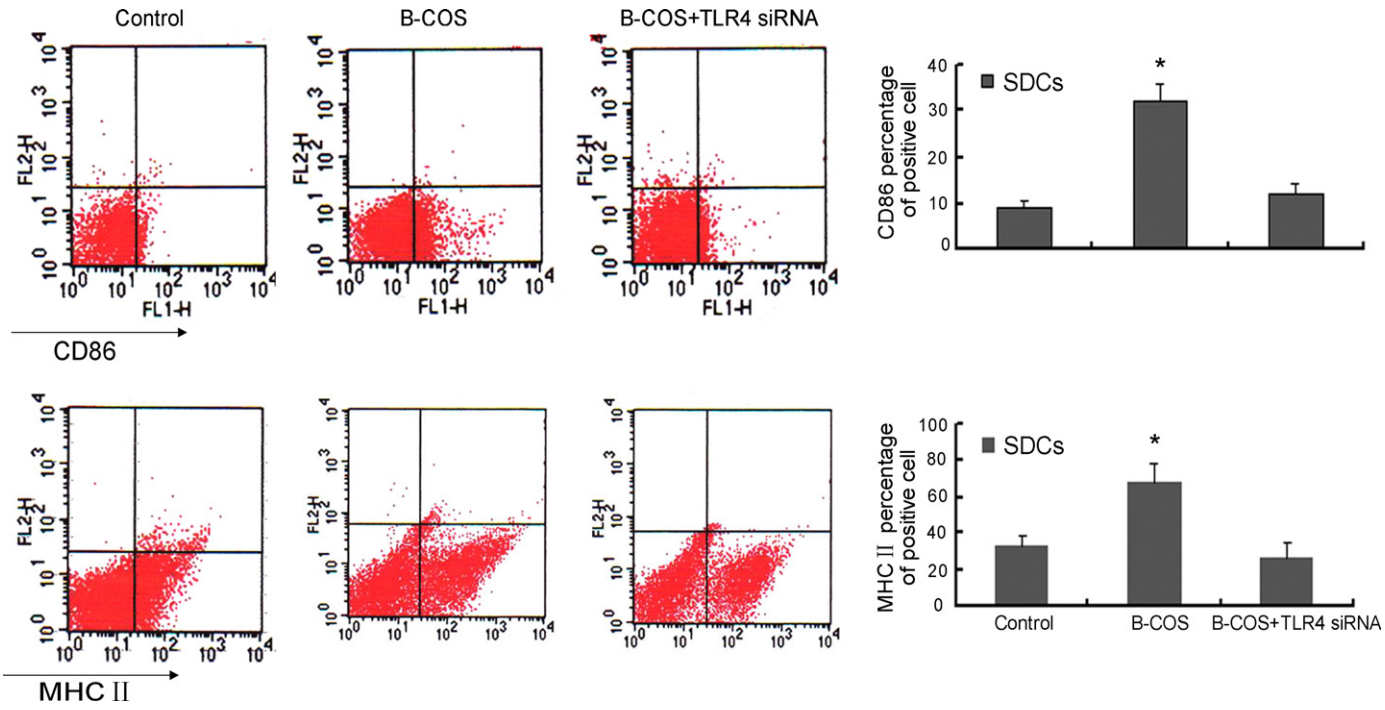

Fig. 5. The expressions of CD86 and MHCII reduced in SDCs with silencing TLR4 treated by B-COS. The results showed that the expressions of CD86 and MHCII were significantly increased under the condition of 160 μg/ml B-COS compared to the control in SDCs before TLR4-specific RNAi. However, CD86 and MHCII were significantly decreased under the same condition after TLR4-specific RNAi.

knock-downed TLR4 expression, compared with that in SDCs without TLR4 RNAi transfection (Fig. 5). The results indicate that B-COS effectively induced the maturation of SDCs and this process may be regulated by TLR4.

3.6. The stimulatory effects of SDCs with silencing TLR4 on the proliferation of T cells declined

To examine the role of TLR4 in SDCs stimulatory capacity induced by B-COS, allogeneic MLR were used in this study. The results showed that B-COS did not promote the proliferation of allogeneic $CD4^+$T cells alone (Fig. 6B), B-COS-treated SDCs obviously promoted the proliferation of allogeneic $CD4^+$T cells (Fig. 6C), whereas B-COS-treated SDCs with silencing TLR4 expression stimulated less proliferation of allogeneic $CD4^+$T cells, compared with SDCs untransfected with TLR4 siRNA (Fig. 6D).

4. Discussion

As mature APCs, SDCs can efficiently activate and induce T cells to secret cytokines, which can regulate both cellular and humoral immune reactions. Furthermore, when activated, SDCs themselves produce many cytokines such as IL-10, IL-12, IFN-γ and TNF-α (Berthier et al., 2003; Hochrein et al., 2001; Stober, Schirmbeck & Reimann, 2001). COS has been shown to markedly modulate the immune system by cytokines (Chen, Wang, Li, & Wang, 2008). Previous papers showed that oligochitosan induced the production of TNF-α and IL-1β in macrophages (Feng et al., 2004). Han et al. found that oligochitosan increased the expression of TNF-α in macrophages (Han, Zhao, Yu, Feng, & Yu, 2005). However, Villiers et al. did not detect significant cytokine expressions in DCs (even no expressions) when treated with chitosan, for example, TNF-α, IL-1β, IL-6, IL-10, IL-12, etc. (Villiers et al., 2009). TNF-α is a central and potent mediator of immune and inflammatory responses (Vassalli, 1992). It is worthy to study whether B-COS can exert some influence over DCs. Our results showed that B-COS, rather than S-COS, increased the expression of TNF-α in SDCs, but no obvious changes of IFN-γ and IL-10 levels were found upon B-COS treatment. There were no statistically significant difference of TNF-α, IFN-γ and IL-10 on the SDCs which were treated by S-COS, compared with the untreated group, respectively. Further research on the regulation of B-COS on SDCs secreting cytokines will be necessary for cytokines serving many functions in immunity.

To date, RNAi technology has generated much insight into the development, activation and function of cells comprising the innate and adaptive immune systems (Mao, Lin, Hung, & Wu, 2007). In addition, RNAi technology has been used to understand the process of antigen presentation by DCs. TLR4 is known to be expressed in macrophages, Kupffer, and DCs (Beutler, 2000; Janeway & Medzhitov, 2002). TLR4 signaling pathways may play an important role in immune cell activation (Kim et al., 2004). Our study demonstrated that the expression of TLR4 could be down-regulated by TLR4 specific siRNA in SDCS, suggesting that this siRNA could be used to further study the role of TLR4 in SDCs.

More recently, (Zhang, Wang, Wu, Jiang & Zheng, 2006) and (Aoki, Ishii, Kanaoka & Kimura, 2006) it has been reported that shRNA targeting TLR4 gene or chemically synthesized TLR4 siRNA can inhibit the TNF-α release by RAW264.7 cells (Aoki et al., 2006). In this study, our results demonstrated that silencing the TLR4 expression on SDCs attenuated the B-COS-induced expression of TNF-α of SDCs. These results suggested that B-COS up-regulated certain cytokine by stimulatory signals and TLR4 was critical for the expression of cytokine.

Immature DCs play a key role in the initiation of adaptive immune responses by capturing antigen in tissues (Steinman & Inaba, 1999). When immature DCs sense danger signals in the environment via PRRs such as TLR, they are induced to mature into efficient antigen-presenting cells (Iwasaki & Medzhitov, 2004). Maturation is accompanied by changes in phenotype such as increased expression of co-stimulatory molecules, and

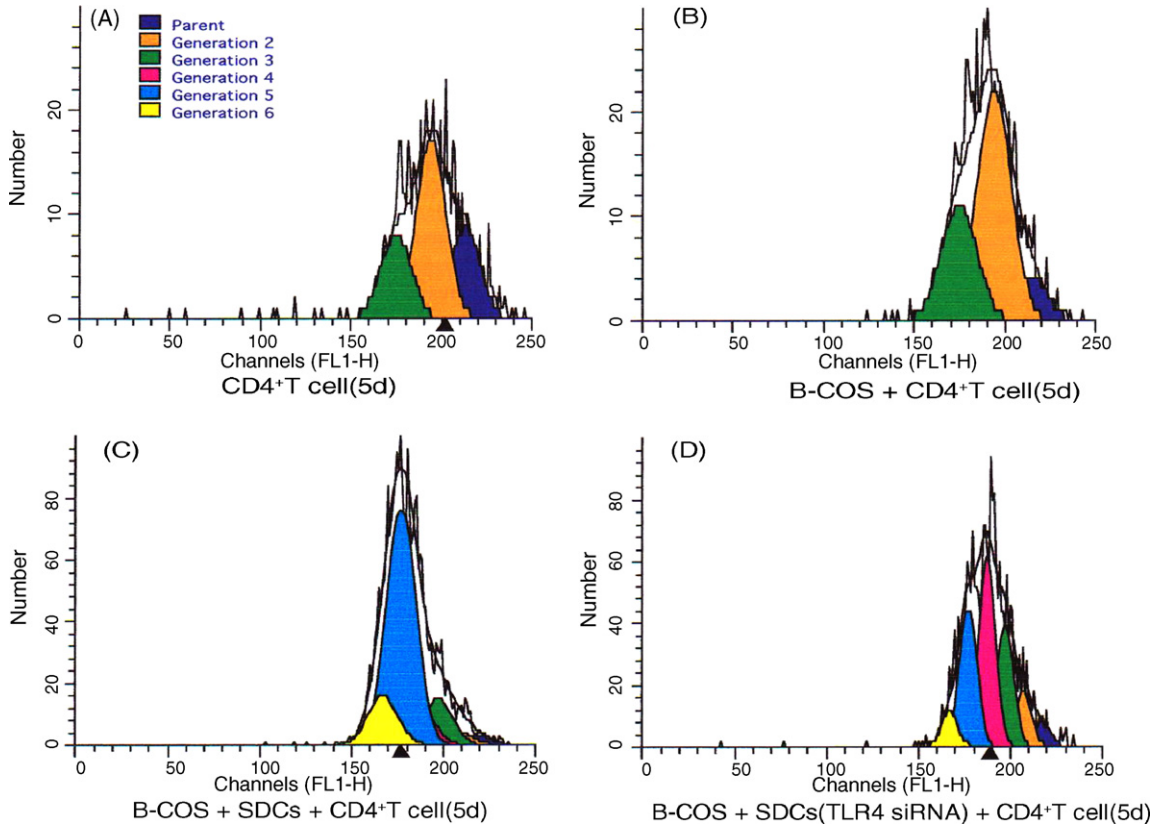

Fig. 6. The stimulatory effects of SDCs on the proliferation of T cells. B-COS did not promote the proliferation of allogeneic $CD4^+$T cells alone (B), but B-COS-treated SDCs obviously promoted the proliferation of allogeneic $CD4^+$T cells (C), whereas B-COS-treated SDCs with silencing TLR4 induced less proliferation of allogeneic $CD4^+$T cells compared with untransfected TLR4 siRNA (D).

MHCII (Napolitani, Rinaldi, Bertoni, Sallusto, & Lanzavecchia, 2005; Villadangos et al., 2001). Furthermore, mature DCs also acquire their ability to activate T cells (Banchereau et al., 2000; Mellman & Steinman, 2001). It was shown that chitosan increased DCs maturation (Porporatto et al., 2005), but some studies reported that chitosan has no effect on DCs (Bivas-Benita et al., 2004; Wischke, Borchert, Zimmermann, Siebenbrodt, & Lorenzen, 2006). Our results demonstrated that B-COS treatment enhanced the surface expressions of CD86 and MHCII on SDCs, rather than TLR4 siRNA-transfected SDCs. Our results also indicated that SDCs treated by B-COS promoted the proliferation of $CD4^+$T cells. But, this stimulatory capacity of SDCs obviously decreased when TLR4 expression on SDCs was silenced by TLR4 siRNA-transfection. These results indicated B-COS could effectively induce the maturation of SDCs and this effect of B-COS may be regulated by TLR4.

The previous paper reported that chitosan induced activation of DCs at the membrane level, but was not able to induce cytokine secretion. This leads to the production of activated DCs unable to stimulate the proliferation of T cells (Villiers et al., 2009). This discrepancy may be due to B-COS and chitosan being different in the molecular size of the glycans, then lead to fairly different SDCs responses.

In conclusion, our results indicated that SDCs could be induced to mature state, secrete TNF-α and promote the proliferation of $CD4^+$T cells by B-COS. TLR4 may play a critical role in this process. The biological activity of COS may be dependent on the molecular size or polymeration degree of COS. But the mechanisms of B-COS recognition and B-COS related signaling pathway are still unknown. We are now trying to identify other receptors for B-COS and the repertoire of downstream pathways activated by B-COS.

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