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RESEARCH ARTICLE

Regulating Activity of Polysaccharides from *Portulaca oleracea* L. on Dendritic Cells of Mice Immunized against Foot-and-Mouth Disease

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A B S T R A C T

Previous study has showed that *portulaca oleracea L.* polysaccharide (POL-P3b), possess adjuvant activity. In the process of immune response, dendritic cells (DC) play a significant role. In view of this, DC maturation and enhancing function were investigated by oral administration with POL-P3b, as an adjuvant for foot-and-mouth disease (FMD) vaccine. The mechanisms involved were further identified. We found that administration with POL-P3b at different concentrations induced DC maturation by increasing co-stimulatory and MHC-II expression. Furthermore, POL-P3b improved stimulus activity of T lymphocyte, and the high dose was more pronounced. In addition, POL-P3b polarized cytokine secretion toward the Th1 pathway. Interestingly, the content of mitochondrial membrane potential was significantly increased in DC+POL-P3b group. To clarify the mechanisms, the effects of POL-P3b on TLR4 signaling pathways were further investigated. Our results showed that POL-P3b treatment increased TLR4 expression and decreased the expression of caspase-3, -9 and -8. On the whole, the experimental results suggested that POL-P3b could be used as an effective adjuvant for FMD vaccine by inducing maturation and enhancing function of DCs, and the mechanism was related to regulating TLR4 signaling pathway.

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INTRODUCTION

Foot-and-mouth disease (FMD) is a serious infectious disease that is apt to occur in cloven hoofed animals. The speed of foot-and-mouth disease spreads very quickly, and epidemic has a certain periodicity. FMD is considered one of the most economically damaging animal epidemics in the world (Cao, 2014). Vaccination is the main way to control FMD in many countries. To better prevent FMD virus infection, adjuvants are often used to increase the vaccine efficacy (Shi *et al*., 2007). Unfortunately, existing adjuvants have many problems such as higher toxicity, local inflammatory response in the injection site and no effective enhancement for cell-mediated immune (Saravanan *et al*., 2015). Growing evidences have demonstrated that Chinese herbal medicine as adjuvants possesses some advantages, including enhancing immune response, lower toxicity and side effects (Feng *et al*., 2015; Li *et al*., 2016).

An ideal new adjuvant should mobilize the body's entire immune function by enhancing the immune response and acting on specific immune cells. Antigen presenting cells play an important role during antiviral immune. Dendritic cells (DCs) are very effective antigen presenting cells, which are considered to be solely responsible for initiating primary immune responses, including antiviral immunity (Steinbrenner *et al*., 2015). Growing evidences have showed that many medicinal herbs as adjuvants could enhance the immune response for vaccine by promoting DCs maturation in animals (Feng *et al*., 2015; Kang *et al*., 2015). These data suggested that the maturation and activation of DC are key event in the process of inducing immune responses to vaccines. Therefore, how to promote DCs maturation and function for FMD vaccine, and thus improve the immunological effect, have attracted wide attention in academic circles.

Portulaca oleracea L., the medicinal and edible plant has been widely used in a lot of countries. In our previous studies, *Portulaca oleracea* L., polysaccharides (POL-P3b) have been found had strengthening body immunity and adjuvant activity (Menendez and Bastida, 2004; Zhao

et al., 2013). However, the activity of POL-P3b for DC maturation states and function as adjuvant for FMD vaccine and the detailed mechanisms remain unclear. The aims of this study were to determine whether oral administration with POL-P3b might promote maturation and function for DC as adjuvant for FMD vaccine and prove whether this effect was related to toll like receptor-4 (TLR4) signaling pathways.

MATERIALS AND METHODS

Mouse GM-CSF recombinant protein, interleukin 4 and interleukin 2 were from R&D Systems; mouse CD11c, MS separation column and MicroBeads were from Miltenyi Biotec, CA; ELISA kit was obtained from Beijing Jingmei biotechnology company. The antibodies used in the experiment were from Santa Cruz Biotechnology, Inc.

POL-P3b and animal immune: POL-P3b were provided according to our previous article (Zhao *et al*., 2013). Forty female BALB/c mice (6-8 w, 18-22 g) were purchased from Changchun medical university. All mice were randomly divided into four groups: a control group (0.9% saline solution), a low dose of POL-P3b (2 μg/mL) group, a medium dose of POL-P3b (10 μg/mL) group, and a high dose of POL-P3b (50 μg/mL) group. All the animals were orally administered daily for 4 days. After 24 h of the last time for oral administration, o-type FMD vaccine was subcutaneously injected in the groin (0.2 mL). After 2 weeks, the same method was adopted to strengthen immunization for one time.

Isolation and culture of DC: Bone marrow-derived DCs were prepared from BALB/c mice. Briefly, the mice were sacrificed and the femurs were used to obtain bone marrow cells by flushing. The individual cells were cultured in the medium containing L-glutamine, 2-mercaptoethanol, rm GM-CSF and rm IL-4. On the day 5, LPS was added into the culture medium. And on the day 6, DCs were collected and MACS was used to isolate DC with CD11c expression by Flowcytometry.

Expression of DC surface costimulatory and MHC-II by flow cytometry: Cells were collected and stained using PE- and FITC-conjugated antibodies. Fluorescence intensities were detected by Flowcytometry.

Determination of cytokine level: Quantification for cytokine using commercially available ELISA kits. The operation process is performed strictly according to the instruction.

Allogeneic mixed lymphocyte reaction: Spleens were obtained from mice under sterile conditions. Cell suspensions were transferred to a nylon fiber column. After centrifuged effluent, the cell pellet was resuspended in RPMI-1640 medium contained rm IL-2 (100 U/mL). DC was cultured in medium using mitomycin C for 60 min at 37°C, then $CD3^+$T cell were added at ratios of 10:1 for 5 d. The proliferation of $CD3^+$T cell was measured by ELISA.

Measurement of mitochondrial membrane potential (MMP): All the experimental cells were incubated in RPMI 1640 added with 0.1 mg/mL rhodamine 123. The MMP was analyzed by flow cytometer. The data of MMP were collected and analyzed using fluorescence intensity (I).

Western blot analysis: Proteins obtained from all the experimental groups were separated using SDS-PAGE, then transferred to nitrocellulose membrane. The different primary antibodies were used to detect corresponding proteins. The secondary antibody was added and incubated at 37°C for 2 h. Immunoreactive signals were visualized using enhanced chemoluminescence reagents (ECL).

Statistical analysis: All the experimental data were expressed as the mean±S.D. Statistical analysis was performed using ANOVA with Dunnett's test, using SPSS10.0 software. $P<0.05$ was considered to be statistically significant.

RESULTS

Effects of POL-P3b on co-stimulatory and MHC-II of DC: DC maturation is characterized by the expression of co-stimulatory and MHC-II molecules. Mature DC is critical for stimulating immune response. Therefore, we evaluated the capacity of POL-P3b to the maturation of DC in mice after immunization. As showed in Fig. 1A and Table 1, oral administration with POL-P3b for mice at different concentrations increased the content of co-stimulatory (CD80, CD86, CD83) and MHC-II, and the expression in high dose group was more significant. Our results demonstrated that POL-P3b could promote DC maturation.

Effect of POL-P3b on cytokine production of DC: Following culture of different groups DC, the cytokine level was detected. The results showed the levels of IL-6、IL-12p70 and TNF-α in DC+POL-P3b (M, H) group were significantly increased compared with DC group (mice immunized with the FMD vaccine alone), but the content of IL-10 was decreased ($P<0.05$) (Fig. 1B). The results demonstrated that POL-P3b as an adjuvant could regulate cytokine production of DC.

Effects of POL-P3b on allogeneic T cell activation of DC: Splenic lymphocyte proliferation is an important indicator for immunity improvement, and mature DC possessed the ability to stimulate proliferation of allogeneic T cells. Therefore, we evaluated the capacity of DC from mice immunized with the FMD vaccine plus POL-P3b to stimulate the T cell response. As showed in Fig. 1C, allogeneic T cells proliferation in POL-P3b (M, H) group was obviously increased compared with DC group ($P<0.05$, $P<0.01$). The experimental results indicated that POL-P3b could enhance T cell stimulatory capacity.

Effect of POL-P3b on MMP of DC: We investigated the effect of POL-P3b on MMP of DC in immunized mice. MMP was significantly increased in POL-P3b treatment group (Fig. 2), compared to the DC group ($P<0.05$, $P<0.01$), which suggested that mitochondria were involved in the function of POL-P3b as an adjuvant on DC.

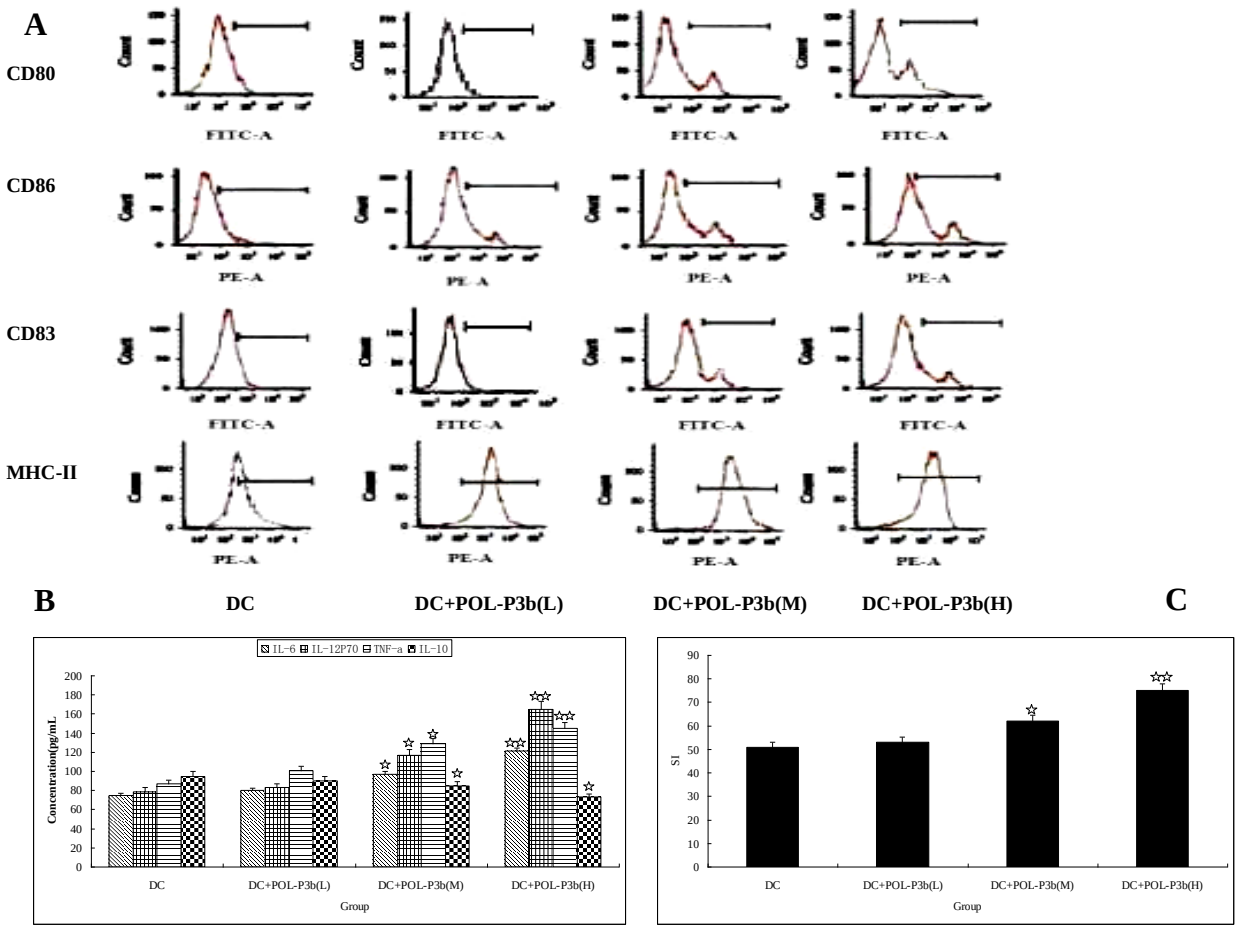

Fig. 1: Effect of POL-P3b on maturation and functions of DC. (A) Phenotypic characterization of DC was analyzed by flow cytometry for expression of the indicated antigens (n=5). (B) Effect of POL-P3b on cytokine production by DC. Data were expressed as mean ± SD(n=10). ☆P<0.05, ☆☆P<0.01, compared with DC. (C) Allogenic T cell activation by POL-P3b-treated DC. ☆P<0.05, ☆☆P<0.01, compared with DC.

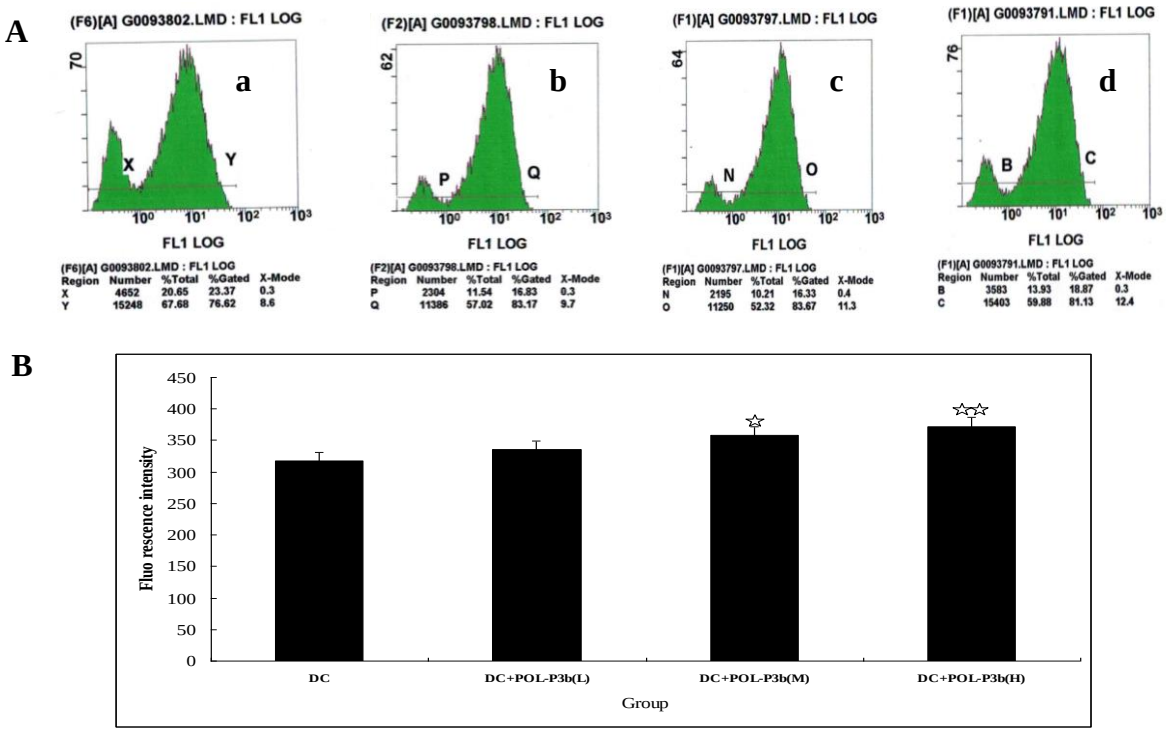

Fig. 2: Effect of POL-P3b on mitochondrial membrane potential (MMP) of DC. (A) MMP of DC was measured using flow cytometer (excitation: 488 nm; emission: 534 nm). Data were also collected in FSC (forward scatter) and SSC (side scatter) and a total of 10,000 events were collected for each sample. a DC group; b DC+POL-P3b (L) group; c DC+POL-P3b (M) group; d DC+POL-P3b (H) group (B) The level of MMP was indicated with fluorescence intensity (I). The computation formula is as follows: I=Log (x-mode)×340. Data were expressed as mean±S.D.(n=10). ☆P<0.05, ☆☆P<0.01, compared with DC.

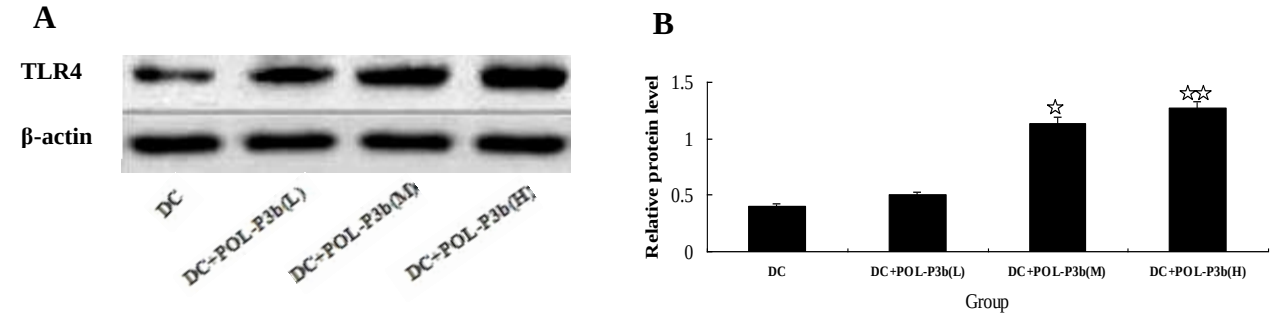

Fig. 3: The effect of POL-P3b on the expression of TLR4 in DC. (A) Western blot analysis showing the expression of TLR-4 in DC treated with or without POL-P3b. (B) Statistical bar graph of expression of TLR-4 protein. Immunoblots were scanned within the linear range and quantitated using the computer software. The quantitated values represent the mean±S.D. compared with DC, $^{*}P<0.05$, $^{**}P<0.01$.

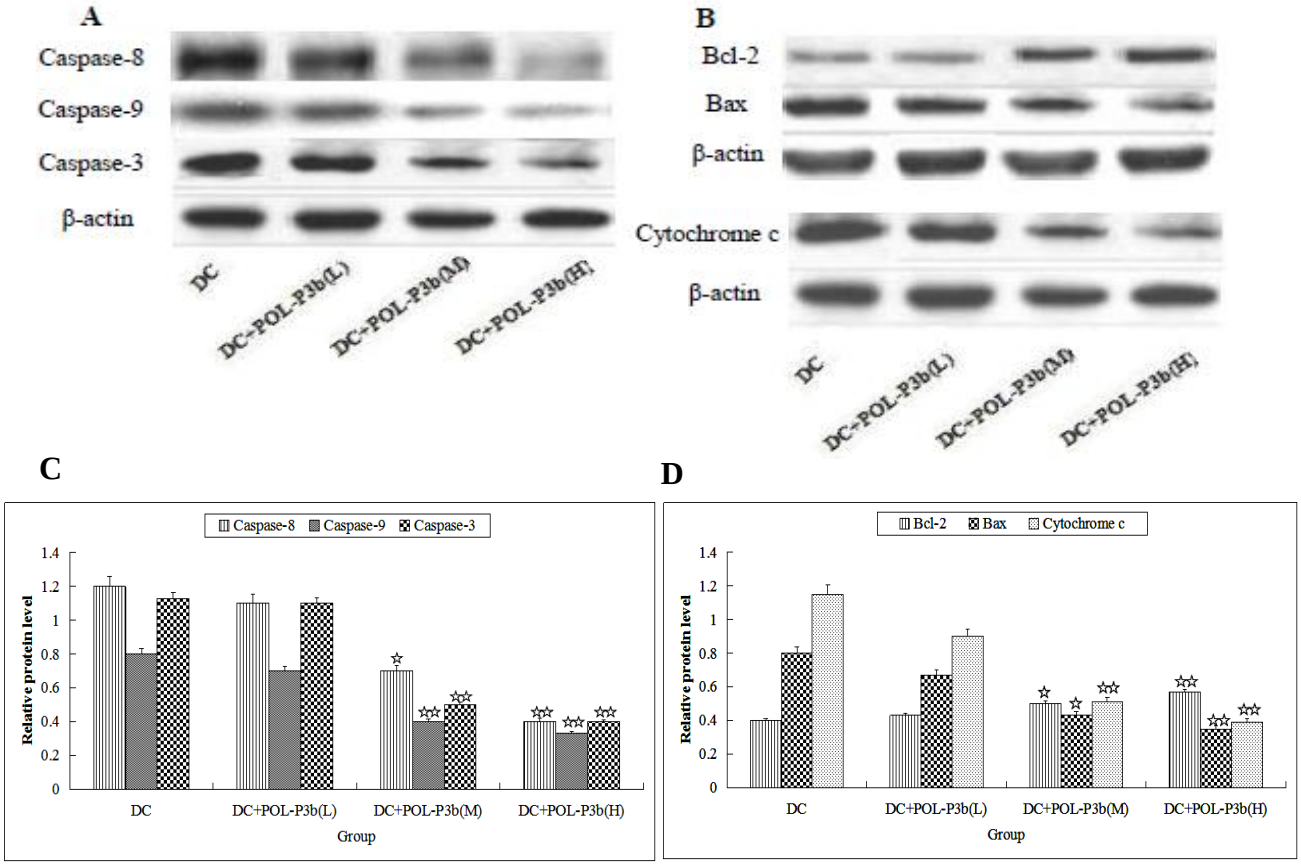

Fig. 4: Effect of POL-P3b on the related proteins of TLR 4 signaling pathway in DC. (A) Expression of cleaved caspase-8, 9, 3 in DC were analyzed by western blot. (B) Expression of Bcl-2, Bax and cytosolic cytochrome c in DC were analyzed by western blot. (C, D) Statistical bar graph of the protein expression. Immunoblots were scanned within the linear range and quantitated using the computer software. The quantitated values represent the mean±S.D. compared with DC, $^{*}P<0.05$, $^{*}P<0.01$, compared with DC.

Table 1: Proportion of positive cells of phenotype expression of DCs in the immunized mice treated with POL-P3b ($\bar{x}$ ±S.D)

Groups	Phenotype expression (%)			
	CD80	CD86	CD83	MHC-Ⅱ
Control (DC)	20.23±1.54	23.64±2.51	14.25±3.15	49.72±5.38
DC+POL-P3b(L)	23.35±1.13	29.73±2.43	14.39±3.05	71.75±5.38*
DC+POL-P3b(M)	37.27±2.07*	35.54±3.13*	20.13±2.38*	90.25±7.39**
DC+POL-P3b(H)	45.51±2.62**	44.73±3.56**	33.73±4.37**	94.39±6.71**

Compared with control, $^{*}P<0.05$; $^{**}P<0.01$.

Effect of POL-P3b on TLR4 signaling pathway in DC: All the above experimental results demonstrated that POL-P3b could promote maturation and function of DC from mice immunized with the FMD vaccine. To investigate further how POL-P3b as adjuvant activated DC and induced their maturation, we first detected the stimulative activities of POL-P3b to TLR4 in DC. The expression of TLR4 was meaningfully increased in DC of mice treated with POL-P3b (Fig. 3). The results indicated that POL-P3b as an adjuvant promote DC maturation and function was concerned with TLR4. To substantiate the involvement of TLR4 signaling pathway, the level of cytochrome c release from mitochondria and the downstream proteins expression were evaluated. An apparent reduction for the cytochrome c content was observed in DC of mice treated with POL-P3b (Fig. 4). We also found Bcl-2 protein was significantly higher, but Bax content was significantly lower in the DC of POL-P3b treatment group. In addition, caspase-3, -9 and -8 activities were significant decrease in the group treated with POL-P3b. All the data suggested that POL-P3b as an adjuvant promote maturation and function in DC occurred through the TLR4 signaling pathway.

DISCUSSION

The mature DC can effectively activate the initial T cells, and it is the center of activation, regulation and maintenance of immune response. Mature DC expresses high levels of costimulatory molecules and adhesion factors. As the most powerful APC, DC can induce specific cytotoxic T lymphocyte (CTL) generation (Salazar *et al*., 2013). Data have displayed that FMDV infection not only decreased the expression of MHC-II, also reduced DC function in the infected host (Cubillos-Zapata *et al*., 2011).

Polysaccharide is the main active ingredient of herbal medicine and has many biological activities including antioxidant, antitumor and immunomodulation (Jin *et al*., 2013). Accumulating evidence has demonstrated that polysaccharides could stimulate the activation of DC by different mechanism (Bo *et al*., 2017; Makarenkova *et al*., 2017). Our previous research has showed that POL-P3b possessed adjuvant activity. In the present study, we main investigated the effect of POL-P3b as adjuvant for FMD vaccine on DC maturation and function. Our results showed POL-P3b could promote DC maturation by up-regulating co-stimulatory (CD80, CD86, CD83) and MHC-II on DC surface. At the same time, POL-P3b could enhance the stimulation of DC to allogeneic T cells proliferation. Our findings revealed that POL-P3b could facilitate DC maturation and enhance its function.

When the body is infected by a virus, Thl/Th2 drift will appear (Zhu *et al*., 2013). Th1 type immune response is a crucial point for antiviral immunity. Importantly, DC could be involved in Th cells differentiation. The mature DC mainly induces immune response to the Thl direction. In the process of Th cells differentiation, cytokine play a role to inhibit or induce Th cells into Th1 cells. The findings showed that POL-P3b stimulated Th1 responses through increasing the secretion amount of TNF-α, IL-12p70 and IL-6. And meanwhile the IL-10 level was decreased. Based on our previous experimental results, POL-P3b as an adjuvant could strengthen T-cell-mediated immune response, and this process was associated with inducing DC maturation and also improving Th1-promoting cytokines release.

The mitochondria are the main energy source for cells, and it is also related to important cell functions, such as apoptosis, movement and signal transduction. MMP is an important indicator for mitochondrial function and its membrane integrity (Park *et al*., 2011). Some studies have demonstrated that changes in MMP might affect DC function by mitochondrial Ca^{2+} accumulation (Patergnani *et al*., 2011; Claire *et al*., 2015). It was interesting to mention that compared with the DC group, the content of MMP was obviously elevated in POL-P3b (M, H) group ($P<0.05$, $P<0.01$). We speculated that POL-P3b influence DC maturation and function was closely correlated with preserving mitochondrial function and membrane integrity. Cytochrome c translocation from mitochondria to the cytosol could be regulated by Bcl family proteins (Carthy *et al*., 2003). In this study, we discovered that POL-P3b increased the Bcl-2 expression and decreased Bax protein content. The effect seemed to be attributed to the inhibition of mitochondrial cytochrome c release by POL-P3b in DC.

DC maturation and activation via TLR4 signaling is critical step in the generation of immune responses. The expression of TLR4 on DC could identify various viruses, and then induce DC maturation and Th1 reaction (Li *et al*., 1997). We found that POL-P3b treatment increased TLR4 expression and decreased caspase-3, -9 and -8 expression. On the whole, all the results suggested that POL-P3b as an adjuvant could induce DC maturation and increase function through stimulating TLR-4 signaling pathway. Interestingly, it has been investigated that several kinds of polysaccharides act as TLR-4 agonists, such as *Ganoderma lucidum* polysaccharides, but the mechanisms are different (Li *et al*., 2015). In conclusion, our data suggested that as an adjuvant for FMD vaccine, POL-P3b could induce maturation and enhance function of DC, and the mechanism was related to regulating TLR4 signaling pathway. This study might offer a theory basis for application of POL-P3b as a novel adjuvant.

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Authors contribution: All authors have made substantial contributions to the manuscript including the conception and design of the study, and acquisition of data, and analysis of data. Also, all authors have approved the version to be submitted. RZ was responsible for design and writing articles. GJ was responsible for preparation of poly-saccharide (POL-P3b) and culture of bone marrow-derived DC. YY was responsible for detection of CD80, CD86, CD83 and MHC-II by flow cytometry. BS was responsible for measurement of mitochondrial membrane potential and data analysis. CH was responsible for detection of protein expression by western blot analysis. LW was responsible for detection of cytokine level by ELISA.

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