Lepista sordida Water Extract Enhances the Maturation of Mouse Dendritic Cells in Vitro and in Vivo

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ABSTRACT

Background: *Lepista sordida* (LS) extract has been shown to possess anti-oxidant, anti-aging, and anti-tumor activities. However, the immunostimulatory effect of LS extract has not been elucidated. **Objective:** To characterize the impact of a water extract of LS (WE-LS) on the maturation and function of mouse dendritic cell (DC) in vitro and in vivo. **Methods:** Mouse bone marrow-derived DCs (BMDCs) were generated. Next, DC maturation was determined by flow cytometry, and cytokine production was measured by ELISA after WE-LS treatment. In addition, DC-induced OVA-specific T cell activation was assayed by [3H]-thymidine incorporation assay. Furthermore, the in vivo effects of WE-LS on DC maturation and Th1 responses in the spleens of mice were assessed by flow cytometry. **Results:** WE-LS treatment up-regulated co-stimulatory (CD40 and CD80) and MHC class II molecules, increased the production of tumor necrosis factor-alpha (TNF-α), IL-6 and IL-12, and enhanced both the proliferation and IFN-γ secretion of allogenic T cells in BMDCs, partially mediated by the TLR2 and TLR4 signaling pathways. Moreover, the in vivo administration of WE-LS to mice enhanced the up-regulation of CD40, CD80 and MHC class II molecules in spleen DCs. WE-LS also increased the generation of T helper type 1 (Th1) cells in vivo. **Conclusion:** These results suggest that WE-LS might have the potential to promote immunity against infection and cancer or to serve as an adjuvant in vaccines and immunotherapies.

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Keywords: Dendritic cells, *Lepista sordida* , Co-stimulatory molecules, Cytokines, maturation

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INTRODUCTION

Dendritic cells (DCs) are efficient antigen-presenting cells and key modulators that link innate and adaptive immunity. They can convert naive T cells towards either immunogenicity or tolerance when exposed to a particular antigen. Following stimulation with microbial stimuli and antigens, DCs migrate to secondary lymphoid tissues and become mature, which greatly enhances their ability to activate T cells (1). Toll-like receptors (TLRs) are the major pattern recognition receptors in DCs and initiate DC function to regulate immune responses via various signaling pathways (2). Because of their key role in immune regulation, DCs offer an individualized approach for therapeutic vaccines to combat cancer and microbial infections (3,4). Substances that induce the activation of DCs may serve as potential adjuvants in immunotherapies and vaccinations.

Growing evidence suggests that many mushroom-derived dietary supplements and bioactive compounds may serve as adjuvants or immune stimulators in the treatment of cancer or infections (5,6). Many compounds have been identified and isolated from mushrooms, with a great potential to be used as nutraceutical and pharmaceutical products in the food industry. Among these compounds, water-soluble polysaccharides and their peptide/protein derivates and various small molecular weight substances are considered to have extremely important roles in immunomodulatory activity and anti-cancer effects (7,8).

Lepista sordida (Fr.) Singer, commonly known as flesh-brown blewit or lilac blewit, is an edible mushroom of the Tricholomataceae family from Asia, Europe, North America, and Brazil (9,10). This small and deep violet-colored mushroom frequently forms fairy rings in grasslands (11,12) and cannot be easily distinguished from *Clitocybe nuda* in the field. Due to the difficulty of its cultivation, only a few successful cultivations have been reported in Taiwan (13), China (14) and Thailand (10). Recently, bioactive extracts from L. sordid have been found to exhibit anti-aging (15), antioxidant (16), and, more importantly, anticancer activities where two distinct groups in China reported that *L. sordida* polysaccharide could lead to downregulations of NF-κB and caspase pathways, contributing to the suppression on the proliferation of human laryngocarcinoma cells (17,18). However, the immunomodulatory effects of *L. sordid* have not been reported.

In this study, we investigated the effects of a water extract of *Lepista sordida* (WE-LS) on the maturation and function of mouse bone marrow-derived dendritic cells (BMDCs) in vitro and *in vivo*.

MATERIALS AND METHODS

Reagents. Recombinant mouse GM-CSF was provided by PeproTech Inc. (Rock Hill, NJ). Fluorescently conjugated anti-mouse monoclonal antibodies (CD11c-PE, CD4-PE, MHC-II-FITC, CD80-FITC, CD40-FITC, IFN-γ-FITC, and IL-4-FITC) and mouse cytokine ELISA kits (IL-12, TNF-α, IL-6, IFN-γ and IL-4) were purchased from eBioscience (San Diego, CA). Lipopolysaccharide (LPS) from E. coli 055:B5, lipoteichoic acid (LTA, LTA; L2515, from Staphylococcus aureus, Sigma, St. Louis, MO, USA) and [3H]thymidine were obtained from Sigma Chemical Co. (St. Louis, MO).

Fungal material identification. Lepista sordida (Supplemental Figure. 1) specimens were collected from the field in Wufeng, Taichung, and the myceliums were purified and cultured on potato dextrose agar (PDA, Difco, Maryland, USA). Next, 100 mg mycelium was removed and homogenized in liquid nitrogen. Genomic DNA was extracted from a 2-week-old culture through the use of a plant genomic DNA kit (GPG1002, Viogene), and then suspended in an elution solution (50 µl). The genomic DNA was amplified by the following universal primer pairs for the ITS region (ITS1, 5.8S rDNA, and ITS2): ITS-A (5'-GGA AGG AGA AGT CGT AAC AAG G-3') and ITS-B (5'- CTT TTC CTC CGC TTA TTG ATA TG-3'). PCR reactions were performed with a program comprised of a hot start at 95 °C for 5 min, followed by 40 cycles at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and a final 72 °C step for 7 min. PCR reactions were performed by Faith BioTechnology Co., Ltd., Taiwan. The 4-µl PCR products were verified using 2% agarose gels (Supplemental Figure. 2) prior to purification and sequencing. The PCR products were then sequenced by Genomics BioTechnology Co., Ltd., Taiwan, with an ABI 3730 XL DNA Analyzer (Applied Biosystems). Comparing the NCBI BLAST results, the DNA sequence was 99% similar to Lepista sordidaaccession no. KF874612.1. In this study, based on NCBI similarity results (Supplemental Figure. 3), the query sequence of the mushroom was shown to be Lepista sordida. The identified Lepista sordida line was cultured (Supplemental Figure 4) and harvested at Taiwan Agricultural Research Institute. The fruiting bodies were immediately frozen after harvest and prepared for freeze-drying.

Preparation of L. sordida Water Extract. After freeze-drying, 30 g dried mushroom sample was milled and extracted employing an aqueous solution in a Soxhlet extractor (40x at 100 °C for 40 min). The extracts were filtered through Whatman no. 1 paper, and the filtrate was evaporated, lyophilized and dissolved in distilled water at 200 mg/mL (stock solution). After that, the extract was passed through an EndoTrap Blue column (Hyglos, Bernried, Germany) in order to remove possible contaminating endotoxins (lipopolysaccharide or LPS). The level of endotoxin in the water extract was measured by a QCL-1000™ Endpoint Chromogenic LAL assay (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA), and was found to be <0.1 ng endotoxin per mg (1 EU/mg) extract. Moreover, to neutralize endotoxins, the samples were incubated with rotation for 2 h at 37 °C with 10 µg/mL polymyxin B (Sigma).

Mice and preparation of bone marrow-derived murine DCs. C57BL/6, C3H/HeN and C3H/HeJ (TLR-4 mutant) mice (6–8 weeks of age) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). TLR-2 knockout mice were provided by Dr. Chih-Peng Chang (NCKU, Tainan, Taiwan). OT-II TCR transgenic mice were provided by Dr. Clifford Lowell (UCSF, San Francisco, CA). All mice were guaranteed to be free of specific pathogens and were housed at the Laboratory Animal Centre at National Chung Hsing University. Approval of the animal care protocols was obtained from the Animal Care and Use Committee of National Chung Hsing University (Number: IACUC-105-074). Murine bone marrow-derived DCs were generated according to methodspreviously described (19). CD11c+DCs were further selected from BM cells with CD11c (N418) microbeads (Miltenyi Biotec), according to the manufacturer's instructions; these cells were utilized for the OVA-specific T-cell activation experiments. The purity of the CD11c+cells was >90%

In vitro phenotypic characterization of DCs. For the in vitro phenotypic characterization of DCs, immature BMDCs were respectively generated from C57BL/6, TLR-2 knockout (TLR-2 KO), C3/HeN and C3H/HeJ (TLR-4 mutant) mice; after that, cells were plated in 6-well culture plates (Corning, Cultek, Madrid, Spain) at 2 x 10^6 cells per well and incubated in 2 ml complete RPMI 1640medium with distilled water (control), WE-LS, LPS (100 ng/ml,TLR4 agonist) or LTA (1 g/ml, TLR2 agonist) at37 °C for 24 h (or for 6 hr for the TNF-alpha ELISA) in a humidified atmosphere containing 5% CO2. Thecells were resuspended in PBS containing 2% FBS, and stained with FITC-labelled anti-MHC-II, anti-CD40, or anti-CD80 plus PE-labelled CD11c antibodies (eBioscience, San Diego, CA,USA), for 30 minon ice. After washing with PBS, the fluorescent intensities were analyzed via an AccuriTM C5 cytometer (BD Biosciences, San Jose, CA, USA) to quantitate the relative mean fluorescence intensity (MFI) changes of molecules on the BMDCs. In addition, the culture supernatants were collected and stored at -80 °C until cytokine examination with sandwich ELISA kits according to the manufacturer's specifications (all from eBioscience, San Diego, CA, USA).

OVA-Specific T-Cell Activation. We employed theprotocol for OVA-specific T-cell activation from our previous report (19). Briefly, spleens from OT-II mice were prepared, and CD4+-positive T cells were isolated using an EasySep Mouse CD4 Positive Selection Kit according to the manufacturer's instructions (Stem Cell Technologies, Grenoble, France). Immature BMDCs were pulsed with 2 μg/mL OVA323–339(OVAP2) (synthesized by Echo Chemical Co., Taiwan) in the presence of WE-LS (100 μg/mL) for 24 hr. The cells were further washed, and OVAP2-specific CD4+T cells (2 × 105) were added to the culture at a DC:T cell ratio of 1:5, 1:10 and 1:25 in 96-well round-bottom plates (Corning). At the end of 96 h, cell proliferation was measured through adding 1 μCi [3H] thymidine(Sigma Chemical Co, St. Louis, MO,USA)for an overnight incubation and quantified by liquid scintillation counting on a β-Counter (Beckman Instruments, Palo Alto, CA, USA). In addition, the culture supernatants were collected, and IFN-γ type-1 T helper (Th1) and IL-4 type-2 T helper (Th2) cytokine levels were specified using sandwich ELISA kits .

In vivo phenotypic characterization of DCs. For the in vivo determination of the effect of WE-LS on DC maturation, C57BL/6 mice (n=6) were injected intraperitoneally (i.p.) with WE-LS at 20 or 100 mg/kg in a total volume of100 μL distilled water once daily for 6 consecutive days. The control group received an equivalent volume (100 μL) of distilled water. Twenty-four hours after the last injection, the mice were sacrificed, and the spleen of each mouse was isolated aseptically and minced using sterile forceps. The cells were then washed twice with PBS, and the expression of maturation (CD40 and CD80) and MHC class II markers was examined by an AccuriTM C5 cytometer. The spleen DCs were identified as lineage-CD11c+cells.Control staining with isotype control IgGs was conducted in all experiments.

Detection of IFN-γ+ and IL-4+ CD4+ T Lymphocytes in Murine Spleens. C57/BL6 mice received i.p. injections of 20 or 100 mg/kg WE-LS in a total volume of 100 μL distilled water once daily for 6 consecutive days. For 6 consecutive days, the control group received an equivalent volume of distilled water once daily. Twenty-four hours after the last injection, splenocytes were harvested and stimulated in vitro for 4 hours with phorbol 12-myristate 13-acetate (50 ng/ml), and ionomycin (1 μM; both from Calbiochem) were added with brefeldin A (10 μg/mL) (Sigma, St. Louis, MO, USA)

during the final 4 hrs. For intracellular cytokine staining, the cells were primarily stained with phycoerythrin (PE)-conjugated CD4+; they were then fixed and permeabilized with Cytofix/Cytoperm buffer (eBioscience) and subsequently stained with FITC-conjugated anti-IFN-γ(type-1 T helper (Th1) cytokine) (eBioscience) and FITC-conjugated anti-IL-4 (type-2 T helper (Th2) cytokine) (eBioscience). The percentages of Th1 or Th2double positive cells among the gated lymphocytes on the basis of forward and side scatter properties or CD4+ T cells were measured by use of an AccuriTM C5 cytometer.

Data analysis. The results are expressed as the means ± SEM. The data were analyzed by one-way ANOVA using GraphPad Prism 5 software (GraphPad Software; San Diego, CA, USA), followed by Tukey's test for multiple comparisons between each group. Student's t-test was used to analyze the data from two groups. P values of less than 0.05 were considered statistically significant. The data are representative of at least three independent experiments

RESULTS

WE-LS activated mouse DCs. Firstly, we tested whether or not WE-LS was able to induce TNFα production in BMDCs. LPS, a known and well-described stimulator of DC maturation, was used as a positive control. The ELISA results indicated that, after WE-LS and LPS (100 ng/ml) stimulation, DCs secreted significant amounts of TNF-α, IL-6 and IL-12, but not IL-4 (Figure 1A) (Figure. 1A). Further examined was the maturation state of DCs after WE-LS stimulation. WE-LS enhanced DC maturation by up-regulating the expression of CD40, CD80, and MHC class II (Figure. 1B). These results demonstrate that WE-LS can activate DCs and promote their maturation.

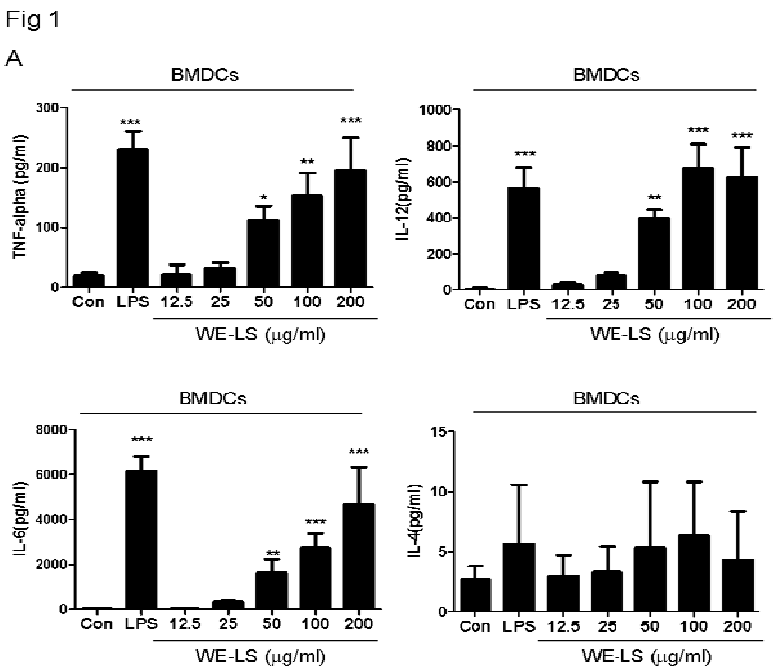
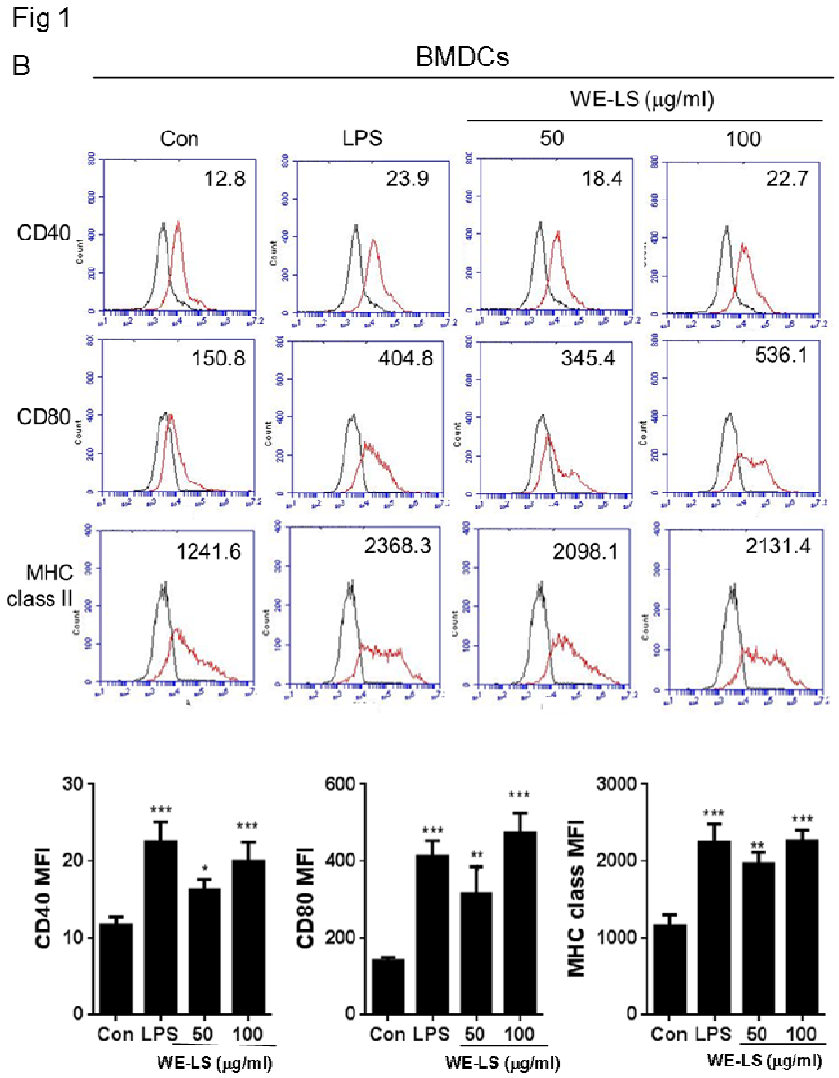


Figure. 1. WE-LS promoted DC maturation and cytokine production. DCs (2×10^6/ml) were treated with WE-LS (12.5-200 μg/ml) for 6 h (for TNF-α expression) or 24 h. LPS (100 ng/ml) was used as positive control. (A) After treatment, the production levels of cytokines in the supernatants were measured by ELISA. (B) The surface expression levels of CD40, CD80, and MHC II on DCs were determined by flow cytometry (histograms). The mean fluorescence intensities (MFIs) of these molecules are



shown statistically in bar graphs. The data represent the mean ± SEM of samples from three wells. *p < 0.05; **p < 0.01 and ***p < 0.001 indicate WE-LS-treated DCs compared to water-treated DCs.

WE-LS enhanced the ability of DCs to induce T cell activation ex vivo. We next tested whether WE-LS-stimulated DCs could activate naïve T cells. OT-II CD4+ T cells were co-cultured with WE-LS treated, OVA323-339 (OVAP2) peptide-pulsed DCs for 96 hours, and T cell proliferation was determined by [3H]thymidine incorporation assays. WE-LS-activated DCs induced more T cell proliferation compared with control cells in vitro (Figure. 2A). In addition, WE-LS-treated DCs increased IFN-γ, but not IL-4 production by T cells (Figure 2B). These results suggest that WE-LS enhances the ability of DCs to induce Ag-specific Th1 cell responses.

Fig 2

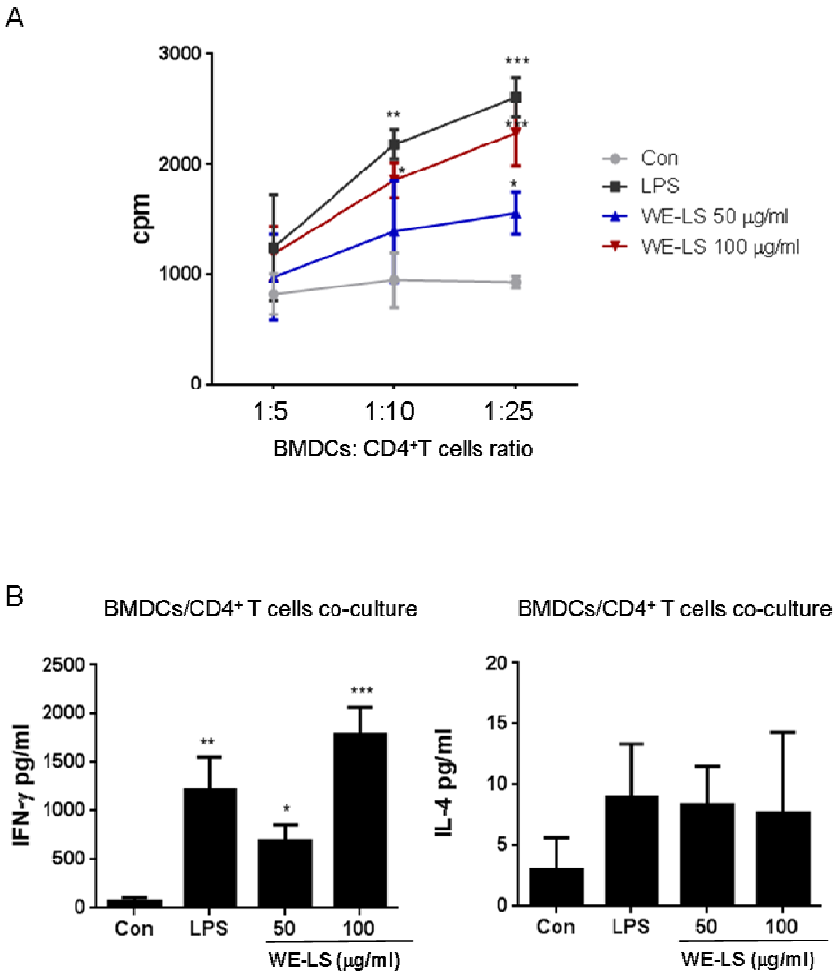


Figure. 2. WE-LS enhanced the DC-induced OT-II specific T-cell responses. (A) The OT-II T cells were co-cultured with water-, LPS- or WE-LS-treated DCs pulsed with 10 μg/ml OVA323-339 peptide at the indicated ratio of DC:T cells for 96 hr. Cell proliferation was specifiedby 3H-thymidine incorporation assays. (B) The supernatants (DC T ratio at 1:25) were harvested, and the amounts of IFN-γ and IL-4 were measured by ELISA. The data represent the mean ± SEM of samples from three wells, and *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ indicate WE-LS-treated DCs compared to water-treated DCs.

WE-LS increased DC maturation through TLR2 and TLR4. To determine whether TLR2 and/or TLR4 are involved in the effects of WE-LS on DCs, we generated BMDCs from TLR2−/− (C57BL/6 background), TLR4-deficient (C3H/HeJ), and wild-type mice (C57BL/6 and C3/HeN) and analyzed cytokine production and co-stimulatory molecule expression. As shown in Figure 3A, LTA (a TLR2 agonist) and LPS (a TLR4 agonist) increased TNF-α and IL-12 production by wild-type DCs, but not TLR2- or TLR4-deficient DCs. Further assessed was the effect of WE-LS on CD80 expression. As expected, WE-LS increased the expression of CD80 in wild-type DCs, but not in

TLR2-deficient DCs (Figure 3B) or TLR4-deficient DCs (Figure 3C). Overall, these data suggest that WE-LS induces BMDC maturation through TLR2 and TLR4 activation

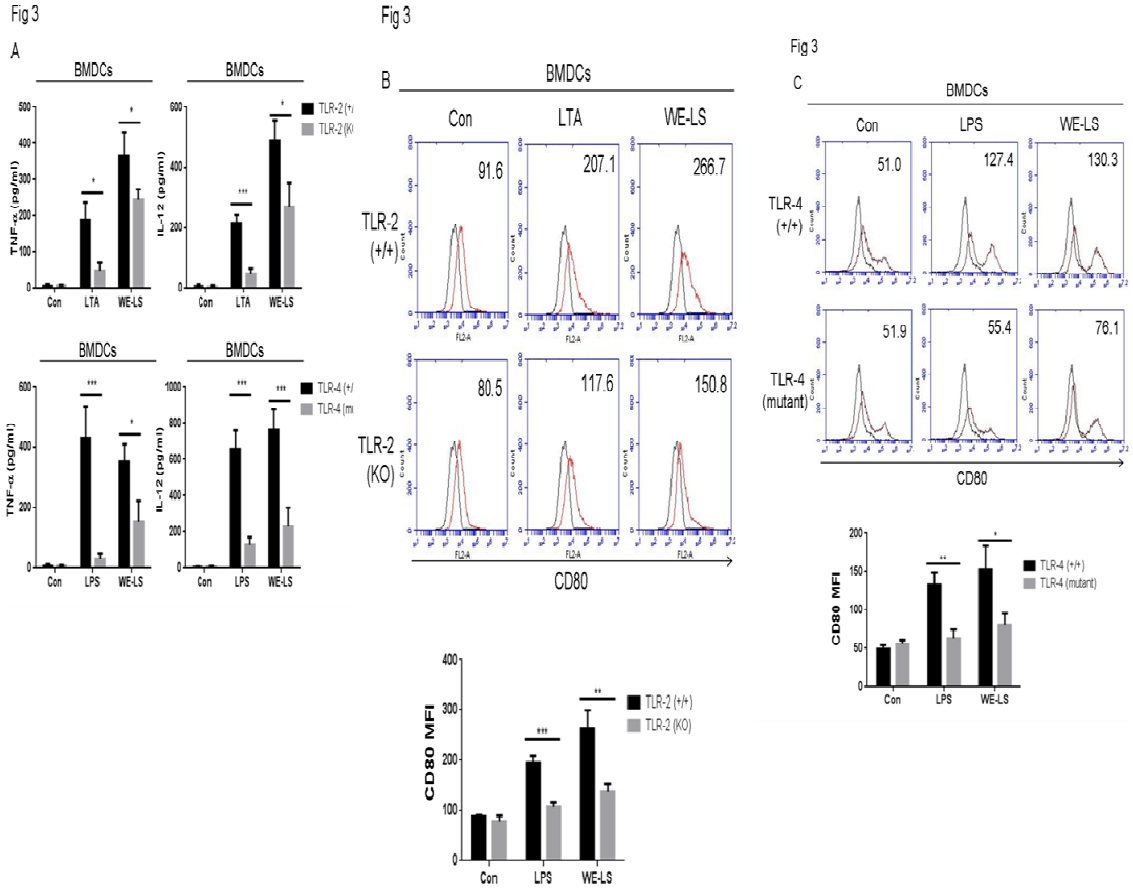


Figure. 3. WE-LS-induced DC maturation and cytokine production were mediated by TLR2 and TLR4. BMDCs were generated from C57BL/6, TLR2 knockout, C3H/HeN, and C3H/HeJ (TLR4 mutant) mice and treated with WE-LS (100 µg/mL), LPS (100 ng/mL) or LTA (1 µg/mL). (A) The production levels of TNF-α and IL-12 in the supernatants were measured by ELISA. (B, C) The expression of CD80 was determined by flow cytometry (histograms). The MFI of CD80 is shown in the bar graphs. The data represent the mean ± SEM of samples from three wells, and $*p < 0.05$; $**p < 0.01$ and $***p < 0.001$ indicate the comparison of stimulated DCs from mutant and wild-type mice.

WE-LS-induced DC activation was not due to endotoxin contamination. Furthermore, to exclude the possibility of endotoxin contamination, BMDCs were incubated with the LPS inhibitor polymyxin B prior to WE-LS stimulation. Polymyxin B did not significantly affect the WE-LS-induced TNF-α and IL-12 production (Figure 4), yet completely inhibited the effect of LPS. These data suggest that WE-LS-induced DC maturation was not due to LPS contamination.

Fig 4

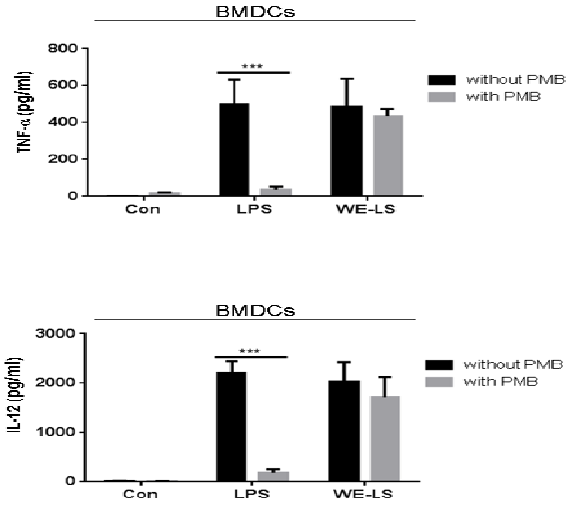


Figure. 4. WE-LS-induced DC maturation was not due to endotoxin contamination.DCs were cultured in 100 μg/ml WE-LS, 100 μg/ml WE-LS + 10 μg/mL polymyxin B (WE-LS + PMB), 100 ng/ml LPS or 100 ng/ml LPS + 10 μg/mL polymyxin B (LPS + PMB). The production levels of TNF-α and IL-12 were measured by ELISA. The data represent the mean ± SEM of samples from three wells, and ***P< 0.001 indicates the comparison of stimulated DCs without or with polymyxin B treatment.

WE-LS induced the activation of spleen DCs in vivo. To further determine whether WE-LS exerted the same effects in vivo, we injected C57BL/6 mice intraperitoneally (i.p.) with WE-LS (20 and 100 mg/kg) for 6 consecutive days, and spleen DC maturation was measured on day 7. As shown in Figure 5, 100 mg/kg WE-LS injection significantly augmented the surface levels of CD40, CD80 and MHC class II in spleen DCs, whereas 20 mg/kg WE-LS had no significant effect. These findings suggest that WE-LS treatment increased the maturation of spleen DCs in vivo.

WE-LS promoted the generation of Th1 cells in vivo. We next examined whether WE-LS can promote the generation of Th1 effector cells. On a daily basis,C57BL/6 mice received 20 or 100 mg/kg WE-LS i.p. for 6 days. On day 7, intracellular IFN-γ, the signature Th1 response cytokine, was clearly up-regulated in CD4 T cells from WE-LS-treated mice; however, the percentages of IL-4-producing CD4 T cells (Th2) were not significantly changed (Figure 6). These data indicate that WE-LS treatment promotes Th1 responses in vivo.

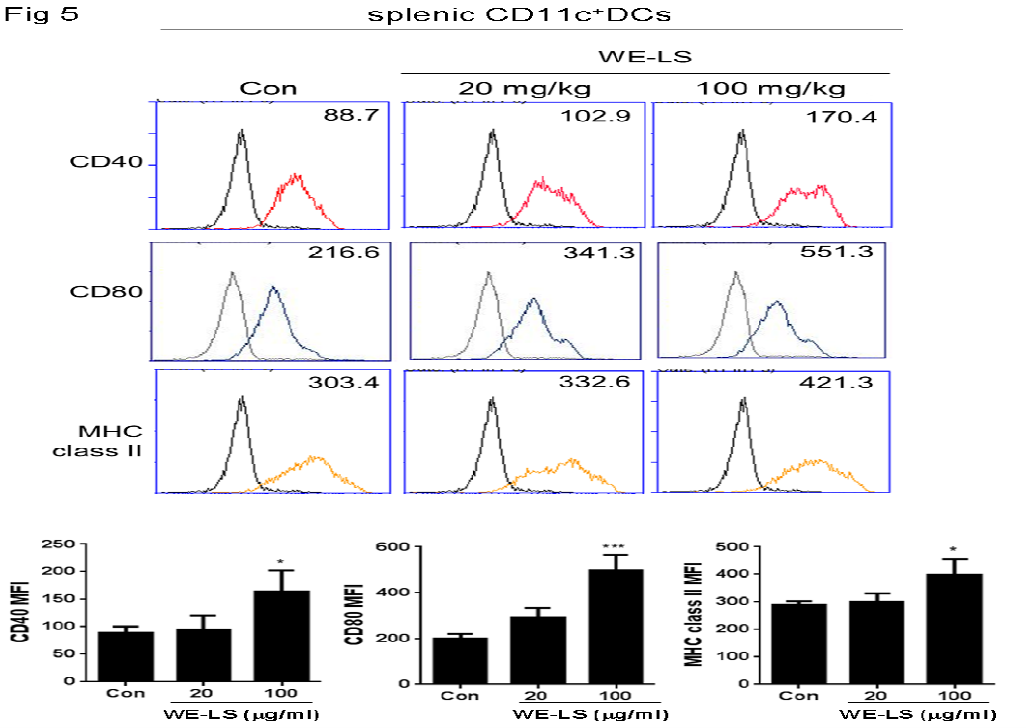


Figure 5. Administration of WE-LS enhanced spleen DC maturation in vivo. C57BL/6 mice were injected intraperitoneally (i.p.) with 20 or 100 mg/kg WE-LS for 6 consecutive days and examined 24 hr following the last injection. The surface expression levels of CD40, CD80, and MHC II on splenic CD11c+DCs were determined by flow cytometry (histograms). The MFIs of these molecules are statistically shown in the bar graphs. The data represent the mean ± SEM of samples from three wells, and *p < 0.05 and ***p < 0.001 indicate the comparison between mice treated with distilled water and WE-LS.

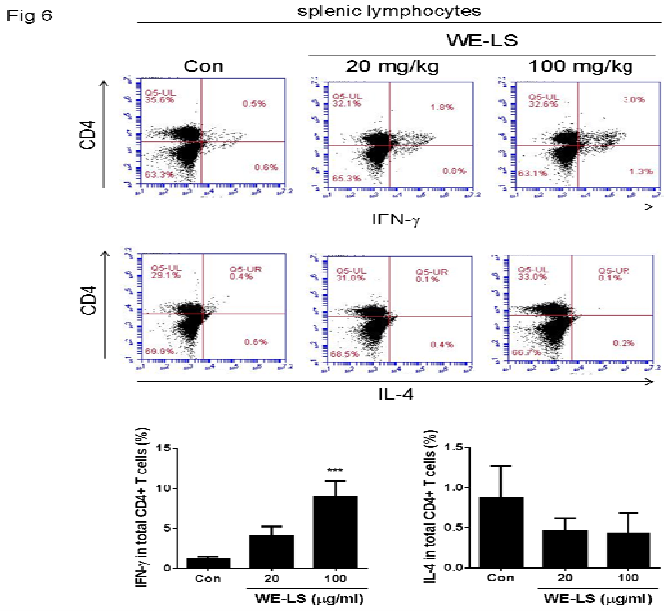


Figure 6. WE-LS increased IFN-γ-producing CD4 T cells in vivo. C57/BL6 mice were injected with 20 or 100 mg/kg WE-LS for 6 consecutive days and examined 24 hr after the last injection. The percentages of IFN-γ- and IL-4-producing CD4 T cells in the splenic lymphocytes were evaluated by flow cytometry (histograms). The percentages of these cells are statistically shown in the bar graphs. The data represent the mean ± SEM of samples from three wells, and ***p < 0.001 indicates the comparison between mice treated with distilled water and WE-LS.

DISCUSSION

Previous studies have shown that liquid extracts from mushrooms can promote DC maturation for cancer treatment (20,21). In this study, we reported, for the first time, that WE-LS strongly up-regulated the expression of CD40, CD80, CD86 and MHC class II molecules on BMDCs in vitro. In addition, the in vivo administration of WE-LS also induced DC maturation in the spleen and Th1 immune responses, suggesting that WE-LS has adjuvant activity, which was confirmed by DC activation.

It has been reported that Th1 immune responses play a major role in anti-tumor immunity, and Th1 polarization is regulated mainly by DCs (22). We report here that WE-LS promoted the secretion of TNF-α and IL-12 by BMDCs, further facilitatingTh1 skewing (23). Consistently, WE-LS-treated DCs significantly induced the secretion of IFN-γ by OVA-specific T cells. Although the increase in Th1 cells of murine splenpocytes did not seem to be as remarkable as expected in our experiment (Fig. 6A), an average 9% of the total CD4 T cells showed IFN-γ+ compared to approximate1% in the control group. However, only about 0.4% of CD4 Th cells exhibited IL-4+, lower than the control mice (~0.9%), in response to the stimulation of L. sordida. Nevertheless, these results demonstrate that WE-LS potentially induces T-cell polarization to the Th1 phenotype.

A number of studies have indicated that TLRs, particularly TLR2 and TLR4, play a crucial role in DC activation by mushroom components, including polysaccharides, proteoglycans and proteins (20,21,24). Accordingly, we examined whether TLR2 and TLR4 were also involved in the mechanism of WE-LS-induced DC maturation. As shown in Fig. 3, DC maturation and cytokine secretion induced by WE-LS, were markedly reduced in BMDCs obtained from TLR2-deficient and TLR4 mutant mice, meaningTLR2 and TLR4 were required for WE-LS-induced DC activation. However, the suppressive effects on maturation and cytokine production were not complete, suggesting that other innate immune receptors, such as other TLRs and C-type lectin receptors (CLRs), may also be involved in WE-LS-mediated DC activation (25-27).

In conclusion, our results provide strong evidence that WE-LS effectively stimulates the activation and maturation of DCs via TLR2 and TLR4, hence the fact that WE-LS is a potential adjuvant for regulating immune responses in vaccines or cancer treatment.

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